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UNIVERSITY OF ALBERTA

Functional Motor Recovery After Nerve Repair

BY

Susan Yufen Fu

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of the Doctor of Philosophy (Ph.D.)

IN

Department of Pharmacology
Division of Neuroscience

Edmonton, Alberta, CANADA
Fall, 1994

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FACULTY OF GRADUATE STUDIES AND RESEARCH

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ABSTRACT

The basis for poor motor recovery after delayed nerve repair is poorly understood. The present experiments examined the effects of immediate nerve implantation onto muscle surface, prolonged motoneurone axotomy and prolonged muscle and intramuscular nerve sheath denervation on nerve regeneration and muscle reinnervation in the rat. Either tibial motoneurones were axotomized or the distal common peroneal nerve sheath and tibialis anterior muscle were denervated for 0 to 365 days. Nerve regeneration was initiated by suture of proximal tibial nerve stump either to the distal common peroneal stump or directly onto denervated muscle surface. At least 6 months later, nerve regeneration and muscle reinnervation were quantified by means of electrophysiological and histochemical methods. After immediate nerve-muscle suture fewer motor units were reinnervated than after nerve-nerve suture. The number of reinnervated motor units also decreased significantly after prolonged motoneurone axotomy and further decreased after prolonged ($>$ 6 months) muscle and intramuscular nerve sheath denervation to less than 10% of the control. However, regenerating axons branched extensively and reinnervated an increasing number of muscle fibres. Consequently, reduction in the number of functional motor units seen in the above three conditions was at least partially compensated. Muscle fibre size was completely restored after immediate nerve-nerve and nerve-muscle sutures and prolonged axotomy after nerve repair. However, long-term denervated muscle fibres gradually failed to recover from denervation atrophy. In conclusion, both prolonged axotomy and prolonged intramuscular nerve stump/target muscle denervation have profound effects on, with prolonged denervation being most detrimental to, functional recovery of motor function.

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LIST OF ABBREVIATIONS

ATPase = Adenosine triphosphatase
CP = Common peroneal nerve
CSA = Cross-sectional area
ECM = Extracellular matrix molecule
EMG = Electromyogram
FF = Fast-fatigable motor unit
FG = Fast-glycolytic muscle fibre
FI = Fast-fatigable intermediate motor unit
Fint = Fast intermediate muscle fibre
FOG = Fast-oxidative glycolytic muscle fibre
FR = Fast-fatigue resistant motor unit
g = Gram
Hz = Hertz
IP = Intraperitoneal
IR = Innervation ratio
Kg = Kilogram
mg = Milligram
μm^2 = Square micrometer
μV = Microvolts
MHC = Myosin heavy chain
mm = Millimetres
mN = Millinewtons
ms = Millisecond
MU = Motor unit
N = Newton
N-CAM = Neural cell adhesion molecule
Ng-CAM = Neural glia cell adhesion molecule
NGF = Nerve growth factor
N-N = Nerve-nerve suture
N-M = Nerve-muscle suture
PAS = Periodic acid-Schiff stain
R-N-N = Ventral root lumber 5 section followed by nerve-nerve suture
S = Slow motor unit
SD = Standard deviation
SE = Standard error
SF = Specific force
SO = Slow oxidative muscle fibre
TA = Tibialis anterior muscle
TIB = Tibial nerve

Chapter 1

General Introduction

1.I...REACTION OF MOTONEURONE TO AXOTOMY

...1...Cell body response
...2...Response of proximal axons

1.II...GROWTH ENVIRONMENT IN THE DISTAL NERVE STUMP
...1...Basal lamina and extracellular matrix molecules
...2...Schwann cells

1.III...CHANGES IN DENERVATED MUSCLE

1.IV...MUSCLE FIBRE PLASTICITY

1.V...SPECIFIC OBJECTIVES OF THE PRESENT STUDIES

1.VI...BIBLIOGRAPHY

The ability of an injured peripheral nerve to restore functional connection with its target was recognized as early as two centuries ago (Fontana 1781 according to Vrbová *et al*. 1994). A century later, Ranvier and Vanlair independently found that restoration of functional connection was due to regeneration of axons from the proximal nerve stump (Ranvier 1874; Vanlair 1882). In this century, the role of distal nerve stump (containing Schwann cells and basal lamina) was identified as providing permissive support for nerve growth and guidance for functional nerve-target connection (Cajal 1928; Nathaniel and Pease 1963; Thomas 1966). The significance of the environment provided by the distal nerve stump is well illustrated by the finding that neuronal axons of the central nervous system (CNS), which do not regenerate after injury, grow into a peripheral nerve stump (Aguayo 1985). Thus, the outcome of nerve regeneration depends upon not only the regenerative capacity of neurons but also the growth environment surrounding the axons.

In spite of the remarkable capacity of injured peripheral nerves to regenerate, functional recovery of the targets is rarely complete (Millesi 1981). This is especially true when nerve repair is delayed (Sunderland 1978, 1991; Merle *et al*. 1986; Birch and Raji 1991). The ultimate recovery of motor function depends upon 1) the number of regenerating motor axons which regenerate and make functional connections with muscle fibres, 2) the number of muscle fibres which are reinnervated, and 3) recovery of muscle fibres from denervation atrophy. The number of regenerating motor axons which successfully reinnervate muscle fibres depends on the nature of nerve injury and repair, the capacity of axotomized motoneurones to survive and regenerate the injured axons, and

the growth support in the environment surrounding both the distal nerve sheath and denervated muscle fibres. The number of muscle fibres which receive reinnervation depends on how many nerve fibres successfully regenerate and reinnervate the target muscle (*i.e.* the number of motor units), how many muscle fibres each motoneurone reinnervates (motor unit size or axonal branching), and the capacity of denervated muscle fibres to survive and remain viable for reinnervation. Provided that all muscle fibres are reinnervated, functional recovery will still not be complete unless denervated muscle fibres fully recover from denervation atrophy.

1.I...REACTION OF MOTONEURONE TO AXOTOMY

1.I.1...Cell body response

A prerequisite for successful nerve regeneration is obviously the survival of neurons that have been separated from their target(s) as a result of injury. The magnitude of neuronal death is affected by a multitude of factors such as age, the degree and location of injuries, and neurone type. After injuries, neuronal death occurs to a degree greater at young age than in adults (Schmalbruch 1984; Himes and Tessler 1989; Snider and Thanedar 1989; Sendtner *et al.* 1990); when the axonal lesion is closer to the cell body; more in sensory and sympathetic neurons than in motoneurones (Aldskogius and Arvidsson 1978). Injured cranial motoneurones are particularly vulnerable to death possibly because the distance between the injury site and cell bodies is short (Arvidsson

and Aldskogius 1982; Yu 1988; Snider and Thanedar 1989). Poor recovery of sensory function may be largely attributed to neuronal death. Similarly, cranial motor function may also be significantly compromised as a result of death of cranial motoneurons. However, mature spinal motoneurones apparently survive after being separated from their targets (Schmalbruch 1984; Melville *et al.* 1989; Gordon *et al.* 1991), even if the separation is permanent (Carlsson *et al.* 1979).

Surviving neurons undergo profound morphological, physiological, and biochemical changes. The main morphological changes include swelling of cell bodies, eccentric nuclei, and dispersal of Nissl bodies due to disintegration of large granular condensations of the rough endoplasmic reticulum (Nissl 1892). The latter phenomenon is often called chromatolysis, which has been suggested to reflect the anabolic response of injured neurons (Watson 1974; Grafstein and McQuarrie 1978).

The onset of regeneration is usually associated with a switch in neurons from a functional transmitting mode to a growth mode, in which the cell's priority for synthesizing neurotransmitters and general maintenance is switched to re-expressing proteins related to axonal growth (Grafstein and McQuarrie, 1978; Gordon 1983). For example, synthesis of tubulin and actin (which are both essential for axonal growth) is greatly enhanced after axotomy (Tetzlaff *et al.* 1991; Matthew and Miller 1993). Other proteins, the synthesis of which is increased, include growth-associated proteins (GAPs) (Skene and Willard 1981; Skene *et al.* 1986). The synthesis of GAP-43, which is a major component of the growth cone, increases 20 to 100 times in regeneration (Skene *et al.*

1986). Although GAPs do not initiate, terminate, or regulate growth, they are necessary for regeneration (Bisby *et al.* 1988). In contrast, there is a decrease in the production of some cytoskeletal proteins such as those comprising neurofilaments, which are normally expressed in development as axons increase in size and form connections with their targets (Hoffman *et al.* 1987; Oblinger and Lasek 1988). The reduction in the synthesis of neurofilament proteins accounts for the decreased diameter of nerve fibres after axotomy and the associated decrease in both the conduction velocity and amplitude of action potentials (Davis *et al.* 1978; Gordon and Stein 1982a; Hoffman and Cleveland 1983; Hoffman 1987; Gordon *et al.* 1991). However, protein synthesis is not necessarily associated with chromatolysis. Some neurons such as sensory neurons in dorsal root ganglia show little or no chromatolysis, but are able to regenerate their injured axons (Hall 1982; Perry *et al.* 1983; Greenberg and Lasek 1988).

The neuronal synthetic machinery is located in the cell body and dendrites, and newly synthesized material for regeneration must be transported to the tip of the axon by axonal transport of fast and slow types. The fast component transports membrane organelles, lipids, and GAPs at a rate of 40-500 mm per day (McQuarrie and Lasek 1989). The slow components are further divided into slow component a (SCa) which transports both neurofilament and microfilament proteins and slow component b (SCb) which transports actin and tubulin proteins at a slightly faster speed (1 to 8 mm/day) than SCa (McQuarrie and Lasek 1989). Other elements transported by SCb include clathrin, fodrin, calmodulin, and glycolytic enzymes which are all essential for axonal growth. After

axotomy, there are changes in the relative amount of some proteins travelling with their specific rate-components. The rate of SCb is 15-40% faster after nerve lesion (Jacobs and McQuarrie 1993; Jacobs and McQuarrie 1992).

It is not known however whether the cell body, when separated from its target for a prolonged time, maintains the capacity to mount sufficient growth associated events for regeneration. Nor is it known whether the rate of axonal transport is maintained in severely atrophied axons after prolonged axotomy. These questions are of considerable clinical importance, such as in a situation where muscle reinnervation may be delayed as a result of severe trauma or long distance between injury site and target (Sunderland 1978). In both cases, the duration of neuronal response to axotomy is inevitably prolonged. The regenerative capacity of long-term axotomized neurons is likely related to the magnitude of the growth-associated response in the cell body.

1.I.2...Response of proximal axons

After injury, axons in the proximal nerve stump undergo traumatic degeneration (Cajal 1928; Sunderland 1978) from the injury site to the first node of Ranvier. In fact, regenerating sprouts originate from the most distal node of Ranvier within a few hours after axotomy (McQuarrie 1985). Formation of regenerating sprouts is too rapid to use the cytoskeletal materials newly synthesized by the cell body which require days to be transported to the tip of axons. Furthermore, growth cones are formed at the injury site even when axons are disconnected from their cell bodies (Shaw and Bray 1977; Mason

and Muller 1982). These findings suggest that the material conventionally available at the axonal tip can initiate formation of the growth cones without the need for newly synthesized material transported from the cell body at least for a short period of time (McQuarrie and Lasak 1989).

If regenerating axons reach the distal nerve stump, each parent axon gives rise to an average of 5 sprouts in the endoneurial tube (Aitken *et al.* 1947; Bray and Aguayo 1974). Although formation of initial axonal sprouts does not rely on the material transported from the cell body, the subsequent rate of axonal growth may largely depend on the rate of SCb (McQuarrie 1983; Wujek and Lasek 1983; McQuarrie and Lasek 1989; McQuarrie and Jacob, 1991). The finding that the regeneration rate in many different types of neurons closely correlates with the rate of SCb strongly supports the concept of the dependence of axonal outgrowth on axonal transport (McQuarrie and Lasek 1989). Axons which have received a conditioning crush two days or earlier prior to a second lesion regenerate sooner and faster (McQuarrie and Grafstein 1973; McQuarrie *et al.* 1977; McQuarrie 1985; McQuarrie and Jacob 1991). The enhanced regenerative response after a conditioning lesion may be attributed to the enhanced synthesis and SCb transport of tubulin, actin, and calmodulin (McQuarrie and Grafstein 1982; McQuarrie 1984; Jacob and McQuarrie, 1993). The minimum effective interval between a conditioning lesion and a test lesion is 2 days and the optimal is 2 weeks (Forman *et al.* 1980). The beneficial effect of a conditioning lesion on axonal regeneration to a second growth stimulus will not be maintained if the interval between the two lesions is beyond 45 days.

In the absence of supportive substrate, regenerating sprouts form a neuroma at the end of the proximal stump (Thomas 1966; Seckel 1990). Neuroma is considered as an expression of the residual growth capacity of the neuron (Young 1948). The diameter of the axons in the proximal stump declines exponentially with increasing duration of axotomy (Davis *et al.* 1978; Gordon *et al.* 1991) and axons remain severely atrophied. Only when reconnected with their target, do the proximal axons recover their size (Gordon and Stein 1982a). It is not clear whether the severe atrophy of axons has any impact on the efficiency of axonal transport and subsequent axonal outgrowth.

1.II...GROWTH ENVIRONMENT IN THE DISTAL NERVE STUMP

Axonal regeneration largely depends upon the interaction between the elongating axon and its growth environment. Normally, regenerating axons are guided to grow in the endoneurial sheath in the distal nerve stump on the route to reinnervate denervated targets. The endoneurial sheath contains Schwann cells and their basal lamina, fibroblasts, and axonal debris if at the early stage of regeneration.

Axons in the distal nerve stump, when separated from the cell body, undergo Wallerian degeneration (Waller 1850). This process includes degradation of the axons and myelin followed by phagocytosis of the debris by invading macrophages and proliferating Schwann cells (review: Fawcett and Keynes 1990; Seckel 1990). The Schwann cell basal lamina persists within the endoneurial tube and maintains columnar orientation of the

proliferating Schwann cells to form the Band of Bungner (Sunderland 1952). Overall, Schwann cells and their basal lamina provide a favourable environment for axonal growth (Schachner *et al.* 1990; review: Fawcett and Keynes 1990).

1.II.1...Basal lamina and extracellular matrix molecules

Schwann cell basal lamina is similar to basal laminae elsewhere and contains extracellular matrix molecules such as laminin, fibronectin, type IV collagen, and heparin sulphate proteoglycan (Junqueira et al. 1986). *In vitro*, laminin and fibronectin have potent effects in supporting neurite outgrowth (Bozycko and Horwitz 1986; review: Letourneau 1988; Bixby and Harris 1991; Carbonetto 1991; Reichardt and Tomaselli 1991; Bixby 1992). The growth-promoting effects of laminin and fibronectin may be mediated by the interaction with their receptor, the integrins, present on the growing axons (Reichardt and Tomaselli 1991). Antibodies against laminin-heparin sulphate proteoglycan complex inhibit neurite outgrowth on sections of peripheral nerves in culture (Sandrok and Matthew 1987). *In vivo*, nerves regenerate in artificial silicone tubes coated with these molecules (Williams 1988; Woolley *et al.* 1990). Peripheral nerve grafts support regeneration even when residing Schwann cells are killed by freezing and thawing (Ide *et al.* 1983; Hall 1986a,b). Muscle grafts are similar to peripheral nerve in their tubular structure of muscle fibre basal lamina and are able to support regeneration by bridging the gap between two injured nerve stumps (Fawcett and Keynes 1986; Glasby *et al.* 1986). Basal laminae of peripheral nerves provide not only potent growth-facilitating components

but also physical guidance for regenerating nerves to reach their targets. This suggestion is supported by the observation that functional recovery is most complete after crush injury in which the basal lamina is intact (Kugelberg *et al.* 1970; Davis *et al.* 1978; Sunderland 1978; Rafuse *et al.* 1992).

1.II.2...Schwann cells

Accumulating evidence, however, shows that basal lamina alone is not sufficient for axonal growth and that Schwann cells are essential in supporting regeneration (review: Fawcett and Keynes 1990). The support provided by Schwann cells is probably not entirely due to their elaborated basal lamina proteins. In culture, Schwann cells provide excellent support for neurite outgrowth even in the absence of their basal lamina (Ard *et al.* 1987). *In vivo*, the nerve does not regenerate through a nerve graft in which Schwann cells are killed (by freezing and thawing) unless Schwann cells have migrated into the graft from the proximal nerve stump (Ide *et al.* 1983; Hall 1986a; Gulati 1988). The same is true for skeletal muscle grafts containing muscle fibre basal lamina and extracellular matrix molecules (Glasby *et al.* 1986b; Fawcett and Keynes 1988). Laminin and some other basal lamina components are potent substrates not only for axonal elongation but also for Schwann cell migration (Le Beau *et al.* 1988; Bailey *et al.* 1993), which may account partly for their role in supporting regeneration *in vivo*. Furthermore, CNS neurons will regenerate their injured axons into the peripheral nerve graft only if viable Schwann cells are present in the graft (Smith and Stevenson 1988). Kormer and Cornbrooks (1985) have

shown that CNS axons only grow along basal lamina tubes which contains Schwann cells instead of basal lamina tubes devoid of Schwann cells. Axonal regeneration is largely compromised when Schwann cells are prevented from migrating into the grafts by cytotoxic agents (Hall 1986b).

Schwann cells elaborate intrinsic cell adhesion molecules (CAMs) which homophilically bind to the same proteins in the regenerating axons to promote axonal outgrowth. Shortly after axotomy, cell adhesion molecules such as L1 and N-CAM are up-regulated in both regenerating axons and Schwann cells in the distal nerve stump (Daniloff *et al.* 1986; Martini and Schachner 1988). *In vitro*, neurites which promote the activity of Schwann cells are almost completely inhibited by antibodies to L1/Ng-CAM, N-cadherin, and integrin, but none alone prevents neurite growth (Bixby *et al.* 1988; Seilheimer and Schachner 1988).

There is extensive evidence that soluble, diffusible neurotrophic factors released from the distal nerve stump can also promote nerve regeneration. Many studies have shown that the denervated nerve sheath can attract regenerating axons and promote intact axons to sprout over a distance of several millimetres (Cajal 1928; Politis *et al.* 1982; Diaz and Pecot-Dechavassine 1990). This is true even when the distal nerve sheath is enclosed in a filter (Kuffler 1986, 1987) suggesting that the diffusible factors are responsible for the attraction. Schwann cells in the denervated nerve sheath are suggested to be responsible for releasing factors that promote axonal outgrowth (Richardson and Ebendal 1982; Ard *et al.* 1985; Schwab and Thoenen 1985).

There are changes in the quality and quantity of many neurotrophic factors and their receptors in the distal nerve stumps and regenerating nerves. Schwann cells in the distal stump produce or express an array of neurotrophic factors and receptors, including nerve growth factor (NGF) and its low affinity receptor (Taniuchi *et al.* 1988; Raivich *et al.* 1991), brain derived growth factor (Richardson and Ebendal 1982; Heumann *et al.* 1987; Ancheson *et al.* 1991), platelet derived growth factor (Raivich and Kreutzberg 1987), insulin-like growth factors (Hansson *et al.* 1986; Kanje *et al.* 1989), and epidermal growth factor receptor (Toma *et al.* 1992). The functional significance of many of these molecular changes in regeneration are yet to be understood. For example, sensory neurons require NGF for survival during development, but they retain little sensitivity to the removal of NGF in adulthood (Lindsay 1988). Motoneurones are insensitive to NGF, although in development they have receptors for it and internalize and retrogradely transport those receptors upon binding with the ligand (Yan *et al.* 1988). It has been suggested that the role of low-affinity NGF receptors in regeneration is to act as cell surface adhesion molecules and NGF links the receptors on the axons and Schwann cells together (Johnson *et al.* 1988).

The endoneurial tube progressively diminishes in size by a factor of 80% to 90% if regenerating axonal sprouts do not enter it (Sunderland and Bradley 1950a,b). The numbers of Schwann cells also decline with increasing duration of denervation (Sunderland 1978; Weinberg and Spencer 1978; Pellegrino and Spencer 1985; Salonen *et al.* 1985). Concurrently, Schwann cell basal lamina disintegrates and eventually disappears (Gianini

and Dyck 1990). In addition, collagen is laid down longitudinally along both surfaces of the basal lamina tube (Sunderland and Bradley 1950a,b). In view of the above changes in the Schwann cell, the Schwann cell basal lamina, and the endoneurial tube, it is likely that growth support in the distal nerve stump wanes with increasing duration of denervation.

1.III...CHANGES IN THE DENERVATED MUSCLE

In addition to the changes in the distal nerve stump after nerve transection, denervated muscle undergoes progressive atrophic changes. The effectiveness of regeneration and reinnervation depends on the location and nature of nerve injury and repair, and the length of time elapsed before regenerating axons reach their target muscle.

After denervation, a rapid initial atrophy results in a 30% reduction in muscle weight in two months (Sunderland 1978). Muscle atrophy decelerates thereafter to reach a stable level by about the fourth month. Reduction in muscle weight is finally stabilized at between 60% to 80% of the normal.

The gross changes in muscle bulk and weight are associated with an even more severe reduction in muscle fibre cross-sectional area. The discrepancy between the reduction in muscle weight and muscle fibre size is due to an increased production of fibrotic tissue in the denervated muscle (Gutmann and Young 1944; Gutmann 1948; Sunderland 1978). In long-term denervated muscle, fatty infiltration and fibrosis are

prominent (Anzil and Wernig, 1990; Schmalbruch *et al* 1991). Fibre cross-sectional area of soleus and extensor digitorum longus in rat decreases to about 3% of the normal by 6 to 10 months after denervation (Schmalbruch *et al*. 1991). As described by Gutmann and Young (1944), muscle fibres after long-term denervation "become reduced to very narrow strands containing a simple series of nuclei". Despite the severe atrophy of muscle fibres, the original endplates are maintained (Gutmann and Young 1944). At later stages of denervation, muscle fibres and endplates also undergo degeneration, necrosis, and regeneration (Gutmann and Young 1944; Anzil and Wernig 1989; Schmalbruch *et al*. 1991). The number of muscle fibres may be maintained initially after denervation by satellite cell proliferation and muscle fibre regeneration as suggested by Anzil and Wernig (1990). Reports of muscle fibre loss in many long-term denervated muscles (Gutmann and Young 1944; Gutmann and Zelena 1962; Anzil and Wernig 1990) are not supported at the ultrastructure level (Schmalbruch *et al*. 1991). Long-term denervated muscles have a normal number of viable muscle fibres which include many regenerated fibres (Schmalbruch *et al*. 1991). Thus, previously reported fibre loss may be due to inability to identify extremely small muscle fibres under the light microscope.

Muscle fibres in long-term denervated muscle contain myofilaments and sarcoplasmic material. However these are not well organised and have few regular cross striations (Schmalbruch *et al*. 1991). Myofilaments become more organised and muscle fibre diameter increases after denervated muscles are electrically stimulated for several weeks (Al Amood *et al*. 1991). This indicates that muscle activity is important in

maintaining the production of contractile proteins. Extremely atrophic muscle fibres still retain the ability to accept reinnervation although regenerating axons tend to form ectopic endplates (Guttman and Young 1944; Anzil and Wernig 1989). However, the severe atrophy of long-term denervated muscle fibres may not be completely reversed by reinnervation (Gutmann and Young 1944; Gutmann 1948; Anzil and Wernig 1989).

In parallel with the morphological changes in muscle fibres after denervation, muscle force decreases rapidly in association with slower contractile speed (Lewis 1972; Kean *et al.* 1974; Finol *et al.* 1981). At later stages of denervation, tetanic tension falls to 10% to 20% in both fast and slow muscles due to gross loss of muscle bulk and a fall in specific tension (review: Vrbova *et al.* 1994).

Changes in muscle fibre structure are also associated with many cellular and molecular changes. The embryonic acetylcholine receptors, which are normally absent from the post-synaptic membrane after innervation, are expressed at the extrajunctional membrane (Miledi 1960; Bambrick and Gordon 1987). N-CAM is up-regulated in denervated muscle fibres (Sanes *et al* 1986; review: Hall and Sanes 1993). The muscle basal lamina components (including laminin, fibronectin and heparin sulphate proteoglycan) are stable after denervation (Sanes *et al.* 1978). N-CAM, together with the extracellular matrix proteins in muscle basal lamina, may account for the observed growth support of denervated muscle sections in culture (Covault *et al.* 1987). Moreover, branching of developing motor axons may be promoted by interaction between growing axons and muscle fibres mediated by N-CAM and L1 (Landmesser *et al.* 1988). Thus,

the increased N-CAM on the denervated muscle fibre surface may promote adhesion between the growing axons and muscle fibres and thereby regulate the branching of regenerating axons in the denervated muscle.

The expression of N-CAM and extracellular matrix molecules are thought to be related to the early development of synapses (Covault *et al.* 1986). These molecules may also be involved in the formation of neuromuscular junctions during regeneration. After denervation, N-CAM and several extracellular matrix molecules such as fibronectin, tanascin and heparin sulphate proteoglycan are expressed in high concentration at the denervated endplate region (Sanes *et al.* 1986; Gatchalian *et al.* 1989). Increased expression of N-CAM is also observed on the muscle fiber membrane after denervation which was suggested to promote axonal growth on denervated muscle in vitro (Covault *et al.* 1987). The junctional fibroblasts which undergo proliferation after denervation are thought to be responsible for the production of the N-CAM and extracellular matrix molecules at the endplate region (Gatchalian *et al.* 1989). These molecules at the endplate region may account for the attraction of regenerating axons by the original endplates which are the preferred sites for reformation of neuromuscular junction (Sanes *et al.* 1978; Kuffler 1986). The attraction of the original endplates is still present even when muscle fibres are removed and only the vacant muscle basal lamina is left (Sanes *at al.* 1978). Although Schwann cells at the junctional region in denervated muscle do not divide after denervation, Schwann cells processes elongate and extend a considerable distance from the

endplate region, and thereby may attract and guide growing axons to the original endplates (Reynolds and Wolff 1992).

It is not known whether long-term denervated muscle fibres continue to express N-CAM and extracellular matrix molecules. Nor is it clear whether junctional Schwann cells and fibroblasts survive and retain their capability of providing growth support and guidance for regenerating nerves.

1.IV...MUSCLE FIBRE PLASTICITY

Regenerating motor axons fail to show specificity at the level of muscle fibres. Although muscle fibres comprising a normal motor unit are typically distributed in a mosaic pattern, regenerating motor axons do not specifically return to connect with the original muscle fibres and often innervate adjacent muscle fibres (Kugelberg *et al.* 1970; Gordon *et al.* 1988; Totosy de Zepetnek *et al.* 1992a). When regeneration favours complete reinnervation of muscle fibres, restoration of normal motor function also relies on re-specification of muscle fibre properties according to those of the new innervating motor axons.

Neural activity has a strong influence on the properties of muscle fibres. This was first observed by Buller *et al.* (1960) who showed in cross-reinnervation experiments that the contractile properties of muscle are regulated by the innervating motoneurones. Since then, a myriad of work has confirmed and extended the concept that many muscle

properties are determined by neural activity (Vrbova *et al.* 1978; Pette and Vrbova 1985; Pette and Vrbova 1992).

Different lines of evidence in support of neural influence come from studies of the properties of muscle fibres innervated by a single motor axon. Normally, muscle fibres of a single motor unit are homogenous in their staining intensity for various muscle enzymes (Edström and Kugelberg 1968; Burke *et al.* 1971; Nemeth *et al.* 1981, 1986; Totosy de Zepetnek *et al.* 1992b) and express homogeneous myosin heavy chain (MHC) isoforms (Gauthier *et al.* 1983; Larsson *et al.* 1991a; Unguez *et al.* 1993). Although regenerating axons do not reinnervate their original muscle fibres, long-term reinnervated muscle fibres in a single MU show the same histochemical profiles (Totosy de Zepetnek *et al.* 1992b). The normal relationship between the amplitude of action potential, MU twitch contraction time, and tetanic force is initially lost after the muscle is self-reinnervated but regained with time (Gordon and Stein 1982).

These findings support the suggestion by Buller *et al.* (1960) that the neural input determines muscle properties. Motoneurones are likely to exert this influence on muscle fibres by imposing the pattern and amount of activity (Vrbova *et al.* 1978; Pette and Vrbova 1985; Pette and Vrbova 1992; Gordon and Pattulo 1993). There is, however, accumulating evidence suggesting that neural control over muscle fibre properties may not be complete. A detailed examination of cross-reinnervation studies suggests that soleus muscles (a slow twitch muscle) retain high endurance and oxidative capacity even after being reinnervated by motor axons formerly supplying fast twitch muscles (Chan *et al.*

1982; Dum *et al.* 1985; Gillespie *et al.* 1987; Foehring *et al.* 1990). In addition, the characteristics of the fast MUs in cross-reinnervated soleus muscles are not identical to those in a normal fast muscle (Dum *et al.* 1985). Also, the variance in the size of muscle fibres of reinnervated motor units is greater than normal (Totosy de Zepetnek *et al.* 1992b). The difference in muscle fibre size among the different histochemical types is reduced after reinnervation (Gordon *et al.* 1988). Some muscle fibres in a cross-reinnervated soleus motor units express both fast and slow myosin heavy chains (Gauthier *et al.* 1983). Recent studies show a heterogeneity in myosin heavy chain isoform expression in muscle fibres within single MUs in aged rat and self-reinnervated cat tibialis anterior muscles providing further evidence for incomplete neural control over muscle properties (Larsson *et al.* 1991; Unguez *et al.* 1993). These findings suggest that muscle fibre properties may be regulated by factors other than nerve supply; these factors may be intrinsic, mechanical, or hormonal (Gordon and Pattulo 1993).

1.V...SPECIFIC OBJECTIVES OF THE PRESENT STUDIES

The objectives of experiments described in the present thesis are as follows:

1) to determine whether the surface of denervated muscle provides sufficient support for neurite outgrowth and branching;

2) to compare the growth support provided by the surface of denervated muscle with that of the intramuscular nerve sheath;

3) to determine whether long-term motoneurone axotomy compromises the capacity of axons to regenerate, to branch extensively to reinnervate many muscle fibre, or to reverse denervation atrophy of muscle fibres;

4) to determine how prolonged denervation affects the ultimate number of motor axons which successfully reinnervate the target muscle and the number of muscle fibres each motoneurone innervates;

5) to determine the capacity of long-term denervated muscle fibres to recover from denervation atrophy following reinnervation;

6) to examine myosin heavy chain isoforms expressed in muscle fibres of single reinnervated motor units and to further test the concept of complete neuronal determination of muscle properties;

7) to determine whether prolonged axotomy or muscle denervation has any impact on the expression of myosin heavy chain isoforms after reinnervation.

1.VI...Bibliography

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CHAPTER 2

Role of the Intramuscular Nerve Sheath in Muscle Reinnervation: an Electrophysiological Study in the Rat Tibialis Anterior Muscle

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2.I...INTRODUCTION

Peripheral nerve axons can regenerate and reinnervate denervated targets (Cajal, 1928). Axonal sprouts originating from the proximal nerve stump are guided into the distal nerve stump by a cellular bridge of Schwann cells (von Bungner, 1891). Once in the distal nerve sheath, regenerating axons normally grow along the interface between Schwann cell surfaces and the underlaying basal lamina (Nathaniel & Pease, 1963; Martini & Schachner, 1988; Martini, 1994). The regenerative capacity of peripheral nerve axons has been attributed primarily to the permissive growth environment in the distal nerve sheath provided by Schwann cells and associated growth supporting molecules (von Bungner, 1891; Aguayo, 1985). Schwann cells synthesize a) both neurotrophic factors and their corresponding cell-surface receptors and b) molecules which act as a substrate for growth (review: Liuzzi & Tedeschi, 1991). These latter molecules may be intrinsic to the cell membrane or secreted to become extracellular matrix (ECM) proteins of the basal lamina (Bunge, Williams & Wood, 1982; Martini, Schachner & Faissner, 1990). The adhesion molecules include the cell adhesion molecules (N-CAM and L1) and the extracellular matrix glycoproteins such as laminin and fibronectin (Martini, 1994).

Although other cell types (macrophages, perineurial fibroblasts) also play important roles in nerve regeneration, Schwann cells are crucial in supporting regeneration over long distances (Fawcett & Keynes, 1990; Vrbova, Gordon & Jones, 1994). However, a pathway of Schwann cells may not be obligatory for peripheral nerve regeneration over

short distance. Axons can regenerate into segments of freeze-killed nerves which initially lack Schwann cells and on surfaces which contain extracellular matrix glycoproteins in culture (Ide, Tohyama, Yokota, Nitatori & Onodera, 1983; Hall, 1986a,b; Bixby, Lilien & Reichardt, 1988). Thus, basal lamina can support nerve growth for a short distance. In addition to nerve sheaths, basal lamina in skeletal muscle can also support neurite outgrowth and branching possibly by providing a substrate for Schwann cells (Glasby, Gschmeissner, Huang & De Souza, 1986; Sanes, Schachner & Covault, 1986).

When regenerating motor axons reach denervated muscle fibres, the axons may grow and branch within intramuscular nerve sheaths (Gutmann & Young, 1944). *In vitro* findings that neurites can grow and branch directly on denervated muscle sections suggest that the high levels of N-CAM and extracellular matrix molecules expressed on denervated muscle surfaces can support nerve regeneration and branching *in vivo* (Covault & Sanes, 1985; Covault, Cunningham & Sanes, 1987). The present study addresses this possibility by asking whether the intramuscular nerve sheaths and their resident Schwann cells are essential for regenerating motor axons to grow into denervated muscles, branch and make functional connections or whether the adhesive molecules on the denervated muscle surface are sufficient. Preliminary results have been published in an abstract (Fu, Tyreman & Gordon, 1991).

2.II...METHODS

2.II.1...Chronic surgery

Young female Sprague-Dawley rats (45-50 days; weight: 150 - 200 g) were anaesthetized with intraperitoneal (*i.p.*) injection of 45 *mg/Kg* sodium pentobarbital. Under aseptic conditions, the right common peroneal (CP) nerve was cut close to the tibialis anterior (TA) muscle and its proximal stump was ligated and sutured to a nearby muscle to prevent its regeneration (Fig. 2.1). All muscles innervated by the CP nerve in the anterior compartment of the hindlimb except TA were removed without damaging nerve or blood supply to the denervated TA muscle. At the same time the posterior tibial (TIB) nerve was cut at the level of ankle and its distal stump was ligated. The proximal stump was sutured either to the distal stump of the CP nerve (N-N suture) or directly to the distal surface of the TA muscle (N-M suture). These two conditions provided two different pathways for nerve regeneration and branching: within the intramuscular nerve sheaths (N-N suture) and outside the sheaths on the denervated TA muscle surfaces (N-M suture). The former group included rats in which the L5 spinal nerve was cut to reduce the number of axons regenerating back to the denervated TA muscle via the intramuscular sheaths (reduced nerve-nerve suture, R-N-N suture) for comparison with the reduced numbers of reinnervated MUs which reinnervated denervated TA muscle after N-M suture (see results).

2.II.2...Acute experiment

Final experiments were performed 9.6±0.5 months (mean±S.E, n=35) after chronic surgery. All rats were fed with 5% dextrose in drinking water for three to five days to load muscle glucose prior to glycogen depletion of a single unit in the reinnervated muscles. Sodium pentobarbital (45 *mg/Kg*) was administered *i.p.* for general anaesthesia with 0.1 *mg/Kg* atropine sulphate *i.p.* to reduce tracheal secretion. Maintenance doses of sodium pentobarbital and atropine diluted in 5% dextrose-saline solution were given via a cannula inserted in the left external jugular vein. The trachea was cannulated for mechanical ventilation when necessary. Heart rate was recorded by an audio-monitor to detect the depth of surgical anaesthesia. Blood volume was maintained by giving 0.5-1 *ml* of the 5% dextrose saline solution via the *i.v.* cannula once an hour.

Laminectomy was performed to expose the L3-L5 ventral roots by removing T13 to L6 vertebrae. As shown in Fig. 2.2A, operated and contralateral control TA muscles were isolated by denervating all hip, tail and other hindlimb muscles. The distal muscle tendon was freed with a small piece of bone and tied with 2-0 silk for attachment to an isometric force transducer. Bipolar surface patch electrodes were sewn onto the muscle fascia for EMG recordings. The bared ends of two teflon coated fine silver wires were inserted alongside the sciatic nerve at a distance of 10 mm to elicit maximal twitch and tetanic contractions.

Both hindlimbs were immobilized at the knees and ankles. The TA muscle tendon was attached to the force transducer for maximal isometric force measurements at optimal

muscle length. A mineral oil pool was prepared around the spinal cord for isolating and splitting ventral root filaments. Rectal and muscle temperatures were monitored with probes and maintained at 35°-37°C and 30°-32°C respectively with a heating blanket and an electric bulb.

2.II.3...Muscle and motor unit force recordings.

Force and EMG signals were amplified, monitored on a Tektronix dual time base oscilloscope (model 5441) and recorded continuously on a Gould 1200S pen recorder. The signals were digitized, averaged on-line and stored on disks with a LSI-11 computer.

Maximal twitch and tetanic forces were recorded in response to single and tetanic trains of stimulation of the sciatic nerve. Two to 5 responses were averaged on line in response to 1, 5 and 21 stimulus pulses at 100 Hz repeated at 1 Hz. Twitch forces of 10-50 MUs were recorded by teasing groups of ventral roots and increasing the stimulus to recruit all-or-none incremental steps in force (Fig. 2.2B). For each increment in force, 2-5 EMG and contractile responses were averaged on-line at 1 Hz (Fig. 2.2C,E). Individual EMG and twitch responses were obtained by digital subtraction (Fig. 2.2D,F). Because this technique is reliable for increments of up to 7 MUs and may overestimate MU force by alternation for larger numbers (see Stein & Yang, 1990), ventral roots were teased to filaments containing 3-8 axons to the TA muscle. At least 30-40% of the MU population was sampled, the smallest number of MUs being studied where n was low and the highest (50) for the largest n.

One filament was selected for isolation of a single motor axon for 1) extensive characterization of motor unit properties, 2) classification into slow (S), fatigue resistant (FR), fast fatigue intermediate (FI) and fast fatiguable (FF) on the basis of contractile speed, "sag" and fatigability and 3) exhaustive tetanic stimulation to glycogen deplete motor unit muscle fibres. The criteria for single MU isolation were an all-or-none twitch contraction and EMG response. A single MU was selected for study only if the threshold voltage of its axons was lower than 6 volt and at least 10 times less than the threshold of any other motor axons to the TA muscle in the same filament. These criteria were rigidly met in order to "hold" the unit for the 1-3 hours of recording required for adequate glycogen depletion. The methods have been described in detail in Totosy de Zepetnek *et al.* (1992b). Briefly short tetanic contractions were elicited by 5 pulses at 50 Hz frequency every second. When the tetanic force had declined to a steady level, the duty cycle rate was increased progressively from 1 Hz to 2 and 3 Hz. When force had fallen to less than 10-20%, the duty cycle was increased to 0.2-0.5 Hz to permit the MU fibres to recover force. This protocol was repeated up to 10 times until the muscle failed to recover 50% of initial force levels during the "recovery cycle".

2.II.4...Histochemistry.

After glycogen depletion, the TA muscle was quickly removed and weighed. Each muscle was cut into 3 cross-sectional blocks along its length and positioned on corks with O.C.T. compound (Tissue teck) for cutting muscle cross-sections. Muscle blocks were

rapidly frozen in isopentane cooled with liquid nitrogen. Cross-sections (each of 10 μm thick) were cut and stained for 1) glycogen, with the use of periodic acid-Schiff (PAS) according to the method of Pearse (1960); 2) myosin ATPase (mATPase) following acid pre-incubation from Green *et al.* (1982); 3) mATPase following alkaline pre-incubation (pH: 10.4) modified from Guth and Samaha (1970), and 4) NADH according to Dubowitz and Brooke (1973) and α-glycerophosphate (α-GP) as described by Gillespie, Gordon & Murphy (1987). Minor changes in buffers and the time period of incubation were adopted in order to distinguish 4 fibre types: slow oxidative (SO), fast oxidative glycolytic (FOG), fast intermediate (Fint) and fast glycolytic (FG) which correspond with S, FR, FI and FF MUs respectively. Details of the methods and fibre typing have been described in Totosy de Zepetnek *et al.* (1992b).

Muscle longitudinal sections, each of 50-100 μm thick, were cut from 4 control and two reinnervated muscles after N-M suture. Intramuscular nerves and motor endplates were visualized on muscle sections by combined silver chloride and cholinesterase stains according to the method described by Kiernan (1990).

2.II.5...Data analysis.

MU number and size. MU number was computed by dividing twitch muscle force by the average twitch force of the MUs (see Jansen & Fladby, 1990). In the example illustrated in Fig. 2.3B, the mean MU twitch force for 28 MUs was 20.1 mN and the

number of MUs was 90. The mean MU twitch force was representative of the 100-fold range in MU force (Fig. 2.3A).

MU number was also calculated by dividing the muscle twitch force by the average MU twitch force for all the muscles in the same experimental or control group for comparison with data from normal un-operated muscles in which 1-3 MUs were sampled in each of the 32 muscles (Totosy de Zepetnek *et al.*, 1992a). Since the number of MUs calculated by the two methods was not statistically different, direct comparisons were made in the MU number between cross-reinnervated and normal un-operated muscles (Fig. 2.4). The relative contribution of innervation ration (IR), cross-sectional area (CSA) and specific force (SF) to MU force were determined to provide a direct conversion factor of MU force to IR.

Muscle fibre number and size. The total number of muscle fibres in each muscle was estimated by multiplying the total CSA of the muscle by muscle fibre density. Muscle cross-sections containing the maximal number of muscle fibres were digitized (JAVA, Jandel Scientific) for measuring muscle CSA and counting muscle fibres in a constant area of 0.63 mm^2 in 6-9 regions of the muscle (a sample of at least 10% of the total number of muscle fibres). Because muscle fibre type and size are differentially represented in different regions between the normal (Pullen, 1977; Parry & Wilkinson, 1991), self-reinnervated (Totosy de Zepetnek *et al.*, 1992a) and cross-reinnervated TA muscles (Fu *et al.*, 1992), representative regions were sampled for measurements of muscle fibre density. Generally small SO and FOG muscle fibres are in the deep medial region and

large Fint and FG fibres are located superficially. However FG fibres in the deep regions are smaller than FG fibres in the superficial region (see Discussion in Totosy de Zepetnek *et al.*, 1992a). Measurement error attributed to differences in muscle fibre size in selected regions was estimated by calculating the number and size of muscle fibres in TA muscles in both hindlimbs of 3 control rats. Differences of up to 10% were due to the differences in fibre size and density in different muscle regions (Table 1).

Average muscle fibre CSA was calculated by dividing the total muscle CSA by total muscle fibre number. In order to validate this estimate, the calculated muscle CSA was compared with the fibre CSA measured directly in 500-1000 muscle fibres in 6-9 representative areas of 4 cross-reinnervated muscles. As shown in Table 2, there was excellent agreement between direct and indirect measurements. The indirect method was therefore used to obtain the average muscle fibre CSA in all the control and cross-reinnervated muscles.

2.II.6...Statistics.

Student's *t* test was used to compare muscle and MU forces and numbers of MUs between cross-reinnervated muscles after N-N and N-M sutures. One-way analysis of variance (*ANOVA*) was applied to examine differences in the same variables between cross-reinnervated muscles after N-N, N-M and R-N-N sutures. Both statistical methods were used for comparisons between cross-reinnervated and control TA muscles. The Kruskal-Willis test of rank order (Sokal & Rohlf, 1981) was used to examine the differences in the

distributions of MU twitch forces amongst different muscles. MU forces of different TA muscles were pooled together for the same experimental condition only if the cumulative distributions were not significantly different. An example in Fig. 2.5 shows the cumulative force distributions of 5 cross-reinnervated TA muscles in which there was an average of 84 ± 10 MUs after N-N suture. Because there was no significant difference in the force distributions, the MUs obtained from the 5 muscles were combined to compare directly with the pooled data from 5 cross-reinnervated muscles which contained a similar number of MUs (88 ± 13) after N-M suture (Fig. 2.6). Kolmogorov-Smirnov Two Sample Goodness of Fit tests (Sokal & Rohlf, 1981) were used to examine the differences between the cumulative distributions of pooled MU twitch forces.

Regression lines were fitted using least square analysis and were drawn through data points only if the value of slopes were significantly different from zero. Arithmetic means were calculated and are shown with standard errors (mean$\pm$S.E). For all the statistical analyses, p values of < 0.05 were regarded as significant.

2.III...RESULTS

A total of 67 rats were used: 32 were un-operated controls and 35 underwent surgery in which posterior tibial (TIB) nerve was cut and sutured either directly to the distal end of CP nerve (N-N suture; n=23) or directly to denervated TA muscle (N-M suture; n=12). For N-N suture, the number of regenerating motor axons was

experimentally reduced in 17 rats by simultaneously cutting the L5 spinal nerve (R-N-N suture). These rats were distinguished from the remaining 6 in N-N suture.

Muscle and MU forces were recorded 90 to 390 days (240±59) after surgery. Since the recovery of muscle force and weight was the same between 90 and 390 days after nerve repair, experimental data were considered together for each operative condition and compared with control muscles.

Muscle force and weight were not different between contralateral TA muscles of experimental rats and TA muscles in age-matched un-operated rats ($p > 0.05$). Thus the data were combined for comparison with cross-reinnervated TA muscles. The MU data used as the control were obtained from the un-operated control muscles.

2.III.1...Muscle reinnervation after nerve-nerve suture.

As shown in Fig. 2.4, the cross-reinnervated TA muscle after N-N suture developed almost as much force as the contralateral un-operated TA muscle. Twitch force was comparable to the control but tetanic force was slightly smaller consistent with the smaller, but not statistically different from the control, recovery of muscle weight. Time to peak twitch force in cross-reinnervated muscles (35.6±1.9 ms) was not different from the control (36.6±1.10 ms) ($p > 0.05$) (Fig. 2.4A,B).

Recovery of muscle force depends on the number of TIB nerve fibres which regenerate and cross-reinnervate the TA muscle (MU number), how many muscle fibres they reinnervate (MU force and N), and reversal of denervation atrophy (muscle fibre

CSA). MU number in normal TA muscles, obtained by dividing muscle twitch force by mean MU twitch force, was 121±6 MUs (n=32) (Fig. 2.4C). After N-N suture, as many TIB nerve fibres reinnervated the muscle (137±23; n=5) and the MUs generated as much force as normal (Fig. 2.4C,D). The small but significant decrease in the number of cross-reinnervated muscle fibres ($p < 0.05$) (Fig. 4E) could account for the small decline in tetanic force and muscle weight (Fig. 4A,B) since the CSA of the cross-reinnervated muscle fibres was not statistically different from normal (Fig. 2.4F).

2.III.2...Muscle reinnervation after nerve-muscle suture.

The cross-reinnervated muscles recovered less well when the TIB motor axons were forced to regenerate outside denervated intramuscular nerve sheaths on denervated muscle surface. Muscle tetanic force (4185±496 mN) was smaller than that after N-N suture (5402±113 mN) (Fig. 2.4A). The decline in muscle force and weight was due to a significant reduction in the number of regenerating TIB motor axons which successfully made functional connections (Fig. 2.4C). Although the regenerated axons reinnervated more muscle fibres than normal with a significant increase in MU force ($p < 0.01$) (Fig. 2.4D), the enlargement of MUs was not sufficient to compensate for the reduction in the number of reinnervated MUs with the result that fewer muscle fibres were reinnervated (Fig. 2.4E). Reinnervated muscle fibres were of same size as normal (Fig. 2.4F).

2.III.3...MU size after N-N and N-M sutures for the same number of regenerating axons.

The ability of regenerating axons to enlarge their MUs after N-M and N-N sutures cannot be evaluated from direct comparison of MU size because of the differences in the number of MUs after two procedures. After N-N suture, the normal number of MUs was associated with MUs which developed forces in the normal range. In order to make a direct comparison of the ability of regenerating nerves to form enlarged MUs after N-N and N-M sutures, the number of reinnervated MUs was experimentally reduced in the N-N suture condition to the same as after N-M suture. This was achieved by cutting spinal nerve L5 which contributed to the TIB nerve when the N-N suture was carried out (reduced N-N suture: R-N-N suture; see Methods).

In 17 rats in which the L5 spinal nerve was cut, the average number of reinnervated MUs (45 ± 9) was the same as after N-M suture without root section (53 ± 11; Fig. 2.4C). In both conditions, average MU force increased 3 fold (Fig. 2.4D). This increase was also associated with incomplete recovery of whole muscle force, weight and the total number of reinnervated muscle fibres (Fig. 2.4A,B,E). Incomplete recovery is consistent with finding that there is an upper limit of 5-8 fold increase in the motor unit size during sprouting of intact motor axons (Brown & Ironton, 1978; Rafuse, Gordon & Orozco, 1992).

Under conditions in which the number of reinnervated MUs was the same after N-M and R-N-N sutures, the range and distribution of cross-reinnervated MU twitch forces were very similar. MU force distributions for each of the 5 muscles after N-M (Fig.

2.5A) and R-N-N (Fig. 2.5B) sutures were not significantly different (Kruskal-Willis rank order test: $p > 0.1$). The data from different rats were therefore combined for comparison of MU force distributions after N-M and R-N-N sutures for the same MU number. No statistical differences were found (Kolmogorov-Smirnov: $p > 0.1$). On the other hand, MU force distributions in the reinnervated muscles was different from normal ($p < 0.01$). As shown in Fig. 2.6, there was a significant shift in force distributions from normal when the MU number was reduced below 50% of normal. The parallel shift of the cumulative histograms along the force axes (Fig. 2.6F,H) indicates that all MUs were enlarged by the same factor; the steeper curve under conditions in which MU number was 15% of normal shows that small MUs increased their forces more than large MUs.

Enlargement of the MU size to compensate for the reduction in MU number had an upper limit of 4-5 times. When the number of MUs was 55% of the normal (76 ± 9), the average MU force increased by a factor of 2 (29.2 ± 2.9 mN and 31.8 ± 2.3 mN after N-M and-R-N-N sutures) (Fig. 2.6A to D). When the number of MUs was reduced to 15% (18 ± 5) of the normal, MU forces increased by a factor of 4 (55.2 ± 3.2 mN and 62.7 ± 7.1 mN after N-M and R-N-N sutures respectively) (Fig. 2.6E-H). The proportional enlargement in MU size is further illustrated in Fig. 2.7 which showed an increase in MU force as the number of MUs decreased. The finding that the slopes of the regression lines fitted for N-N (including R-N-N) and N-M sutures were not different ($p > 0.1$) showed that regenerating motor axons have the same capacity to enlarge MUs to

compensate for the reduction in MU number. However, for both conditions recovery of muscle force deteriorated as the number of reinnervated MUs decreased (Fig. 2.8).

2.III.4...Innervation ratio and patterns.

In order to obtain a more direct measure of MU size, muscle fibres of a single MU were glycogen depleted for enumeration and measurement of fibre CSA. Twitch and tetanic forces correlated well with the number of muscle fibres per motoneurone (N) and to a lesser degree with the mean MU fibre CSA (A) and the calculated specific force (S). When each variable was plotted on double logarithmic scales as a function of force, the slope of each line provided a measure of the relative contribution of each variable because muscle force is the product of the three variables, namely F=NAS (Fig. 2.9; also see Stein, Gordon & Totosy de Zepetnek, 1990; Totosy de Zepetnek *et al.*, 1992a). It is clear from the greater slope of regression of force with N than with A and S that N was the major determinant of muscle force in reinnervated muscles. Furthermore, the relationship between force and N was the same for twitch and tetanic forces.

These results and the comparison of MU force, N and computed IR (muscle fibre number/MU number) showed that MU twitch force measurements were a good indicator of MU size in terms of the number of muscle fibres reinnervated by each regenerated axon (Fig. 2.10). The variance between rats was primarily due to the variance in the number of MUs under the two experimental conditions. The variation after R-N-N was due to the high degree of variability in the contribution of each ventral root to the innervation of

hindlimb muscles (Gordon, Stein & Thomas, 1986). The variance after N-M suture, on the other hand, probably reflects the variability in the distance over which regenerating motor axons must grow to reinnervate the denervated endplates directly on the surface of the muscle fibres or indirectly by growing toward and entering intramuscular nerve sheaths (see Discussion below).

As shown in Fig. 2.11, regenerating motor axons after N-M suture reinnervated denervated muscle fibres at the original endplate zones. There was no evidence for ectopic endplate formation.

Normally muscle fibres of a single MU intermingle with fibres of other MUs but occupy a distinct area or MU territory (Fig. 2.12A; Totosy de Zepetnek *et al.*, 1992a; Gordon, Yang, Stein & Tyreman, 1993). The pattern of axonal branching, which cannot be directly assessed, is reflected by the spatial distribution of glycogen depleted MU fibres in cross-sections of the reinnervated muscles showing that regenerating axons branched in a more localized area than the normal (Fig. 2.12B to D). After N-N suture, MU territory was smaller than normal and the majority of MU fibres clumped (Fig. 2.12B). A similar spatial distribution was found after R-N-N suture (Fig. 2.12D) where the number of regenerating nerves was experimentally reduced by cutting one spinal ventral root. However, each MU included more muscle fibres than normal. Because all denervated muscle fibres were not reinnervated, the cross-sectional area of the reinnervated muscles was reduced. After N-M suture, MUs were enlarged and the spatial

distribution of muscle fibres was very similar to those after N-N and R-N-N sutures (Fig. 2.12C).

2.IV...DISCUSSION

The results of the present study demonstrate that denervated muscle surface is a relatively poor substrate for nerve growth as compared to intramuscular nerve sheaths. Yet, at least for small muscles, regenerating motor axons which are forced to grow outside the intramuscular sheaths can branch and reinnervate as many denervated muscle fibres as when they regenerate back to the denervated muscle via the normal route through the intramuscular nerve sheaths.

2.IV.1...Regeneration: intramuscular nerve sheaths and denervated muscle surface.

To address the question whether denervated muscle fibres and their basal lamina can support nerve regeneration and branching, nerves were forced to regenerate outside the intramuscular nerve sheath on the surface of denervated muscle. This was achieved by suturing TIB nerve to the denervated TA muscle surface far from the normal CP nerve entry point without causing strain on the suture. The comparable recovery of self- and cross-reinnervated TA muscles after N-N suture (Totosy de Zepetnek *et al.*, 1992a) indicates that a foreign (TIB) nerve is as successful as the original nerve in reinnervating the muscle.

Significantly fewer regenerating motor axons made functional connections when these grew outside the intramuscular nerve sheaths than within the nerve sheaths. Thus, even though acellular nerve and muscle grafts have supported nerve regeneration over short distance (Hall 1986a,b; Gulati, 1988; Fawcett & Keynes, 1990), the present findings demonstrate that denervated muscle surfaces are inadequate growth substrates as compared to the intramuscular nerve sheaths with their Schwann cells. These cells and their associated growth-promoting molecules provide an environment more conducive to axonal regeneration (Aguayo, 1985; Bunge & Hopkins, 1990). Schwann cells either express the cues as intrinsic constituents of their cell membrane (such as N-CAM and L1) or secrete the cues as extracellular matrix molecules such as fibronectin and laminin (Bunge & Bunge, 1983; Schachner, Antonicek, Fahrig, Faissner, Fischer, Kunemund, Martini, Meyer, Persohn, Pollerberg, Probstmeier, Sadoul, Sadoul, Seilheimer & Thor, 1990).

Nevertheless, the observation that some motor axons did regenerate and make functional connections after N-M suture indicates that denervated muscle surfaces, although not as adequate as intramuscular sheaths, can support axon elongation to some extent. Furthermore, since these axons made connections at the original endplate sites, the denervated muscle surface must also provide axonal guidance cues. One possible reason for the success of a smaller number of motor axons in making muscle contacts is that the denervated muscle surface provides an adequate substrate for some but not all the motor axons. A more plausible explanation is that the axons grow along the denervated muscle fibres to reach denervated intramuscular nerve sheaths and, where possible, re-

enter the sheaths as their preferred pathway of growth. Experiments in amphibians have shown that axons implanted into denervated muscles will grow preferentially to denervated nerve sheaths and that this attraction is mediated by a soluble factor released from non-neural cells contained in the sheath (Ard, Bunge & Bunge, 1985; Schwab & Thoenen, 1985; Kuffler, 1986, 1987; Grinnell 1988). Even when the normal innervation of a muscle is intact, an implanted nerve sheath provides a potent stimulus for collateral sprouting of intact nerves (Torigoe, 1985; Diaz & Pecot-Decjavassine, 1990).

Even if regenerating motor axons are attracted to the intramuscular sheaths as their preferred growth pathway, the surgical implantation of the TIB nerve at least 10 mm from the original nerve entry point and 4 mm from denervated endplate sites in the present experiment means that regenerating motor axon are forced to use the denervated muscle surfaces for a significant proportion of their elongation. Muscle sheaths can support nerve regeneration over small distance since muscle has been used as interpositional grafts in experimental nerve repair (Glasby, Gschmeissner, Hitchcock & Huang, 1986; Fawcett & Keynes, 1990) and denervated muscle has been shown to support neurite outgrowth *in vitro* (Covault *et al.*, 1987).

It is likely that the high levels of N-CAM on the denervated muscle fibre membrane and the continued expression of ECM molecules such as fibronectin and laminin provide a permissive substrate for the growth of some regenerating axons. However, the high failure rate of muscle reinnervation after N-M suture suggests that either the denervated muscle surface supports fewer regenerating axons or the distances over which the axons

can elongate is limited. Since regenerating axons must traverse muscle fibres to reinnervate denervated endplates, many will encounter the connective tissue barrier of the perimysial sheaths which may hinder further elongation. Normally the motor axons traverse muscle fibres within intramuscular sheaths and do not encounter these barriers except at terminal branches. The majority of growing axons are likely to make functional connections via the intramuscular sheaths which guide the nerves back to the endplate regions of the muscles. This argument could also account for the high variability between animals in the number of regenerating axons which were successful in reinnervating TA muscle after N-M suture. Even a small difference in the location of nerve implantation would change the distance over which the nerve must grow on the denervated muscle surface to reach intramuscular sheaths or endplate sites. Since the TIB nerve was implanted distal to the endplate zone, regenerating axons would likely enter the most distal regions of the intramuscular nerve sheaths. If the distance over which regenerating axons can elongate is small, it can be expected that the farther the location of implantation away from the intramuscular sheaths and the endplate sites, the fewer axons would reinnervate the TA muscle. In support of this argument, significantly more regenerating axons make functional connections when nerve implantation was close to the original nerve entry point (Gordon & Stein, 1982; Gillespie, Gordon & Murphy, 1986).

The original endplates remained the preferred site of formation of nerve-muscle junctions even when axons were forced to regenerate outside the intramuscular nerve sheaths (Fig. 2.11). Regenerating axons grew back to the endplates either through the

nerve sheaths after re-entry or directly on denervated muscle surface. Both the guidance by intramuscular sheaths and attraction by basal lamina components at the endplate region are important since regenerating axons make contacts at original endplate sites even when muscle fibres have been destroyed (Sanes, Marshall & McMahan, 1978; Hall & Sanes, 1993). Junctional Schwann cells which extend elaborate processes and migrate for a short distance (Reynolds & Woolf, 1992) may contribute to the attraction, guidance and support for regenerating axons to motor endplate sites. This hypothesis is supported by findings that ectopic endplates are formed on denervated muscles when the sheath is not denervated prior to nerve implantation (Frank, Jansen, Lomo & Westgaard 1975) or has deteriorated after long-term denervation (Gutmann & Young, 1944). Ectopic synapses may also be induced by localized muscle damage (Sayers & Tonge, 1984).

2.IV.2...Branching and MU formation: nerve sheaths and denervated muscle surface.

The smaller number of axons which made functional nerve-muscle connections after N-M suture branched extensively to supply an average of 3 times more muscle fibres than normal. This contrasts with the normal size and number of MUs in the cross-reinnervated muscles after N-N suture (Fig. 2.4). MU size could be 5 times as large as normal after N-M suture which is the same upper limit for sprouting from uninjured nerves (Brown & Ironton, 1978; Rafuse *et al.*, 1992). This increase in the number of muscle fibres per motoneurone accounts for the significantly larger MU forces in the cross-reinnervated muscles after N-M suture.

When the number of regenerating axons was experimentally reduced at the same time as N-N suture, MU size increased to the same extent as in N-M suture. Thus, regenerating axons have the same capacity as uninjured axons to increase their MU size to compensate for a reduction in MU number and, at least in the rat, the increase in MU size occurs whether or not axons grow in the intramuscular sheaths. However, the ability of regenerating axons to form enlarged MUs depends on the size of the muscle, the spatial distribution of MU fibres and the relative number of proximal and distal nerve branches. In rat hindlimb muscles, the size of the territory occupied by MU fibres is small as compared to the larger territories in the larger cat hindlimb muscles (Rafuse & Gordon, 1994a). After self- or cross-reinnervations, MU fibres clump within a smaller territory in rats and fibre type grouping is obvious (Fig. 2.12; see also Kugelberg, Edstrom & Abruzzese, 1970; Totosy de Zepetnek *et al.*, 1992a). This spatial distribution indicates that most axonal branching occurs in the terminal regions of the sheath. In contrast, little clumping is seen in MU fibres or fibre types in reinnervated cat muscles after N-N suture (Gordon, 1987; Nemeth, Cope, Kushner & Nemeth, 1993; Unguez, Bodine-Fowler, Roy, Pierotti & Edgerton, 1993; Rafuse & Gordon, 1994a) unless MU number is dramatically reduced (Rafuse & Gordon, 1994a,b). This suggests that branching occurs more proximally in the intramuscular sheaths in larger muscles. It is likely that the sheath is essential for branching in these large muscles because 1) MU fibres may be several millimetres apart from each other and in different fascicles and 2) MUs fail to enlarge after N-M suture to compensate for reduced number of reinnervated MUs (Rafuse & Gordon,

1994a,b). Clearly, terminal branching is sufficient to distribute nerve terminals to denervated muscle fibres in small rat TA muscle in contrast to larger cat muscles.

The consistent finding that MU enlargement is limited accounts for the observation that neither muscle force nor muscle size fully recovered in cross-reinnervated TA muscles after N-M suture. A 3-fold increase in MU size could not fully compensate for a reduction in the number of MUs to less than 30% of the normal.

A parallel shift of the cumulative force distributions to larger forces after R-N-N and N-M sutures suggests that all axons enlarged MUs (Figs. 2.9-2.11). Since MU force and IR are normally correlated with axonal size, these observations corroborate our earlier findings that the size of all MUs is increased by the same proportion and small axons still supply fewer muscle fibres than large axons (Rafuse *et al.*, 1992). A tendency for the cumulative force distributions to become steeper for large increases in MU size suggests that the smaller axons may increase in IR somewhat more than expected (Figs. 2.9 & 2.10).

2.IV.3...Conclusions.

When regenerating axons are forced to grow outside the intramuscular nerve sheaths, the number of axons which succeed in making functional nerve-muscle connections is dramatically reduced. However the capacity of regenerating axons to branch and reinnervate more muscle fibres than normal is not compromised with the result that the increase in MU size compensates, to some extent, for the reduction in the number

of functional MUs. Even though regenerating axons may have used the denervated muscle surface as a growth substrate, intramuscular nerve sheaths provide better attraction and support for growing axons. Under conditions in which axons grow on denervated muscle surfaces, the nerve sheaths may still be the preferred pathway through which axons grow and branch. The reduction in the number of axons which succeed in making functional connection is attributed to their failure to grow towards the sheaths and/or denervated endplates (where terminal connections are preferentially made). Such failure reflects the sub-optimal capacity of denervated muscle surface to support nerve elongation in contrast to the conducive growth environment within intramuscular nerve sheaths.

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Table 2.1. Comparison of indirect measurements (means ± S.E.M.) of muscle fibre number in the tibialis anterior muscles between left and right sides in control rats.

	Rat 1		Rat 2		Rat 3	
	Right	Left	Right	Left	Right	Left
Fibre Density (#/mm^2)	316 ± 44	284 ± 33	340 ± 31	288 ± 39	244 ± 29	273 ± 39
Muscle CSA (mm^2)	41.8	38.8	37.6	36.2	49.3	42.4
Fibre Number	13219	11019	12777	10426	12029	11560

Note: CSA = Cross sectional area

Table 2.2. Comparison of direct and indirect measurements (means ± S.E.) of muscle fibre cross-sectional area (CSA).

	Muscle 1	Muscle 2	Muscle 3	Muscle 4
Direct ($\mu m^2 \pm$S.D.)	2445 ± 822	2980 ± 1052	3026 ± 1350	2941 ± 1213
Calculated (μm^2)	2500	3394	2894	3154

Fig. 2.1

Diagrammatic illustration of normal innervation of the tibialis anterior (TA) muscle, nerve-nerve (N-N) suture, and nerve-muscle (N-M) suture. A: The sciatic nerve normally branches into the common peroneal (CP) nerve and posterior tibial (TIB) nerve. CP nerve innervates muscles in the anterior compartment of the hindlimb including the tibialis anterior (TA) muscle (darkened). TIB nerve innervates the intrinsic muscles of the foot (not shown). B: The TIB nerve was cut and its proximal stump was sutured to the distal stump of the cut CP nerve to cross-reinnervate the TA muscle. C: The CP nerve was cut and its proximal stump was sutured to biceps femoris muscle to prevent its regeneration. In the same operation, the TIB nerve was cut at the level of ankle and its proximal stump was sutured directly to the surface of the denervated TA muscle as far as possible from the original entry point of the CP nerve to the muscle (at the tip of V shaped endplate zone).

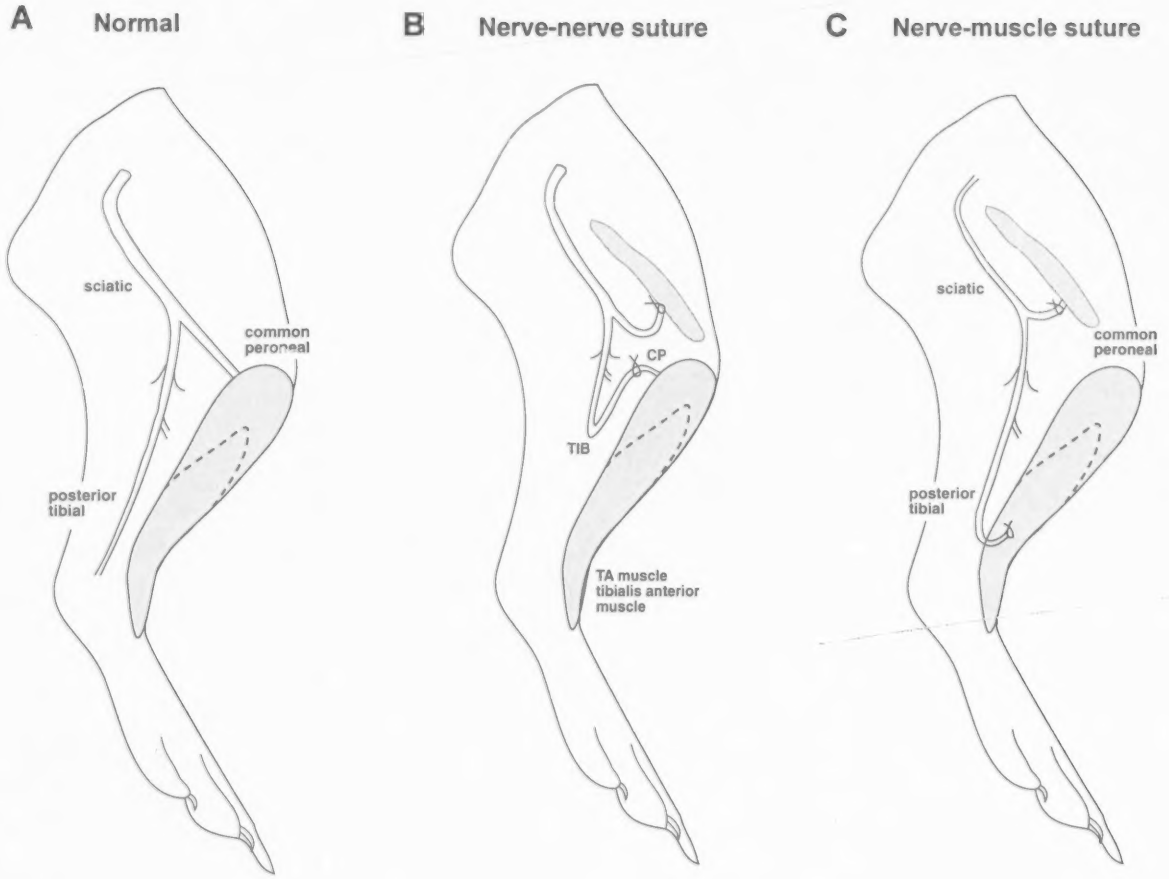

A
Normal
sciatic
common peroneal
posterior tibial
B
Nerve-nerve suture
CP
TIB
TA muscle tibialis anterior muscle
C
Nerve-muscle suture
sciatic
common peroneal
posterior tibial

Fig. 2.2

Muscle, motor unit (MU) force and EMG recordings. A: Tibialis anterior (TA) muscle was isolated by denervating all other hindlimb muscles and attached to a force transducer. Ventral roots L5-L6 were isolated and split for stimulation of single motor axons contributing to TA muscle. B: Chart records of force increments in response to incremental stimulation of a teased ventral root filament. C: All-or-none EMG signals elicited from 3 MUs: solid line for MU #1, dashed line for MUs 1 and 2, and dotted line for MUs 1,2, and 3. D: Single MU EMG signals obtained by digital substraction (*e.g.* unit 2 EMG=units 1 & 2 EMG - unit 1 EMG) among the same three MUs in 2C. By the same method, all-or-none MU twitch force increments (E) and single MU twitch forces (F) were obtained.

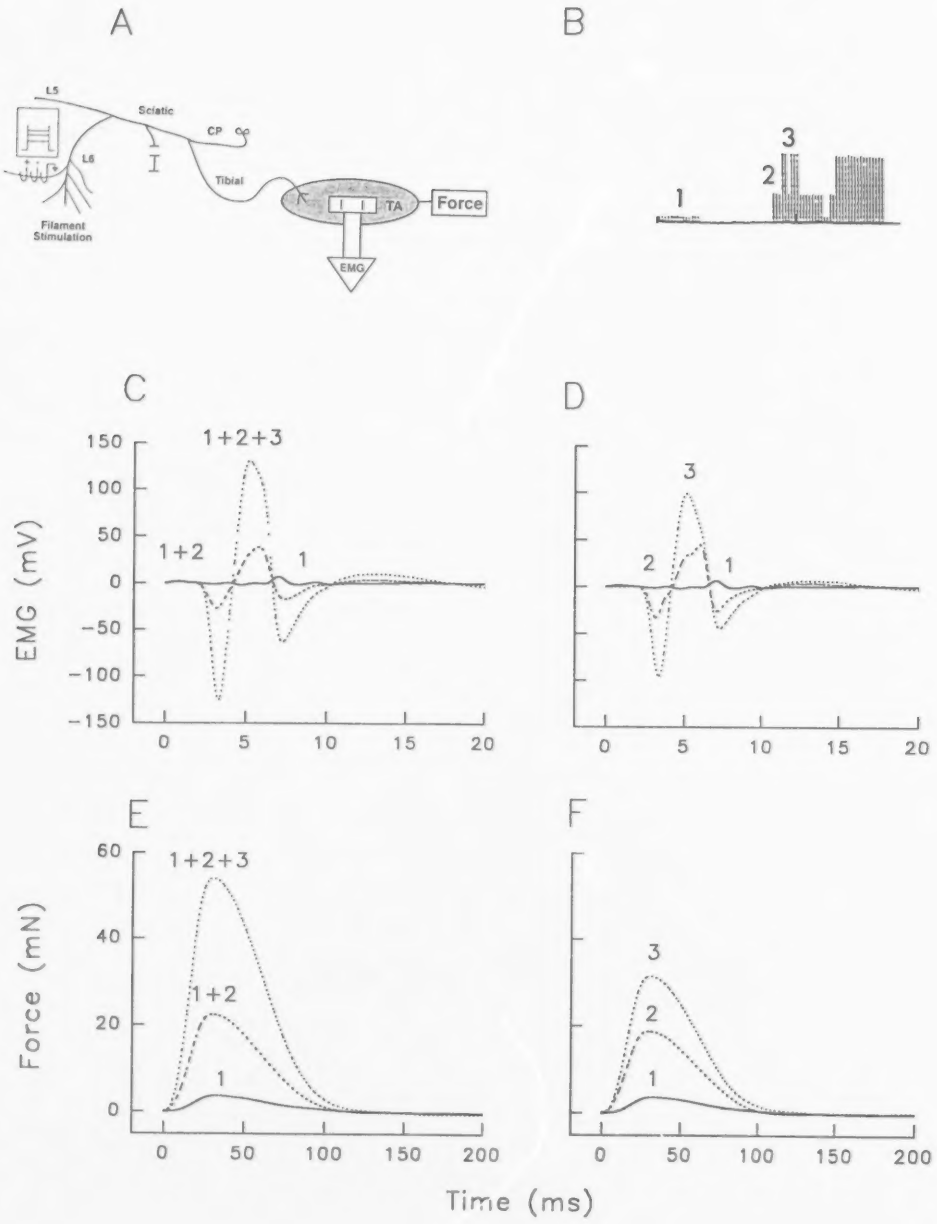
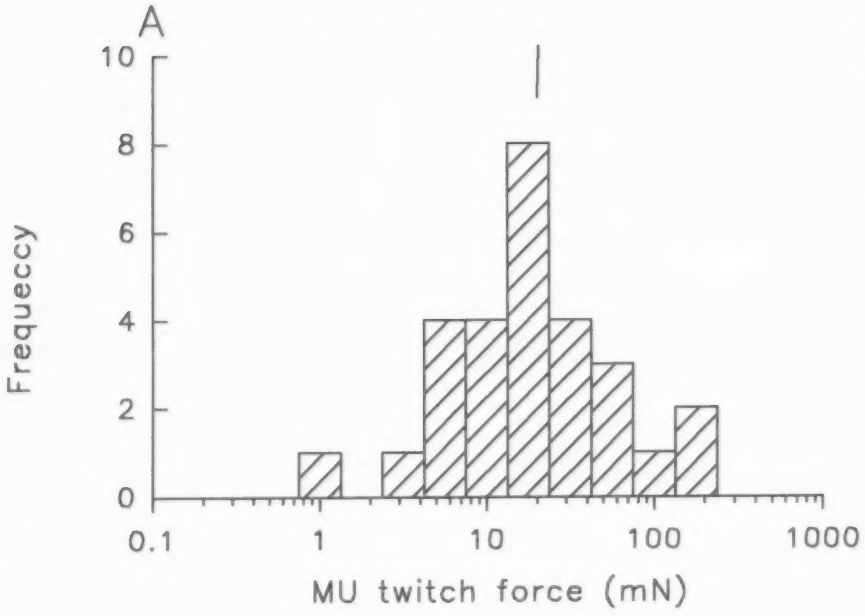

A
B
L5
Sciatic
CP
L6
Tibial
TA
Force
EMG
Filament Stimulation
1
2
3
C
D
1+2+3
1+2
1
2
3
EMG (mV)
150
100
50
0
−50
−100
−150
0
5
10
15
20
E
F
1+2+3
1+2
1
2
3
Force (mN)
60
40
20
0
0
50
100
150
200
Time (ms)

Fig. 2.3

Distribution of MU twitch forces and the method used to calculate the number of MUs. A: Frequency distribution of MU twitch forces sampled from 28 MUs in a typical example of a reinnervated TA muscle. Twitch forces were normally distributed in a 100-fold range (mean$\pm$S.E.: 20.1$\pm$5.7 mN). B: Twitch force (1809 mN) was divided by mean MU to give the MU number of 90.


A
10
8
6
4
2
0
Frequeccy
0.1
1
10
100
1000
MU twitch force (mN)

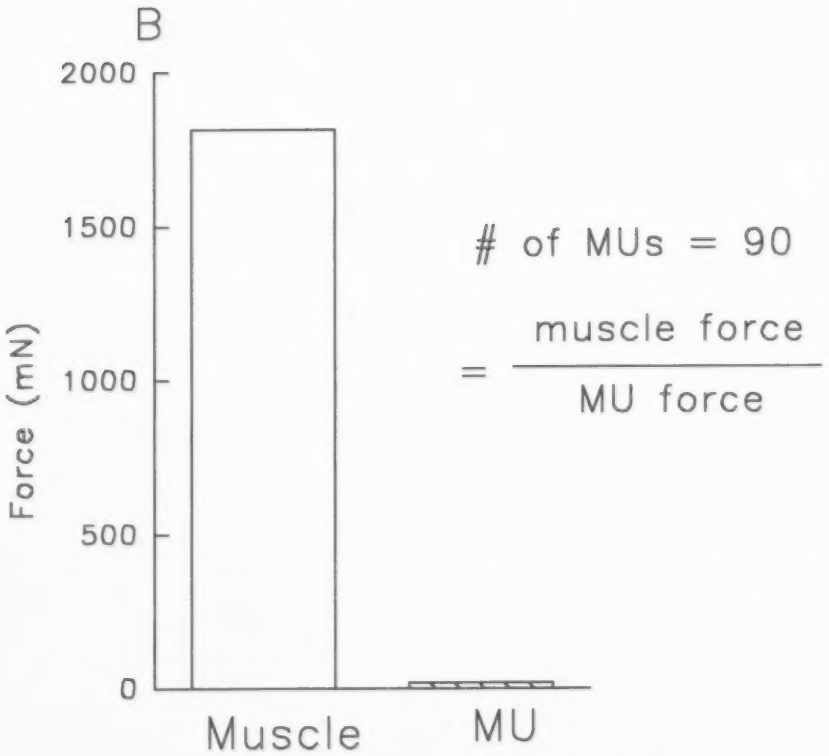

B
2000
1500
1000
500
0
Force (mN)
Muscle
MU
# of MUs = 90
= muscle force / MU force

Fig. 2.4

Mean (±S.E.) muscle tetanic force (A), muscle weight (B), number of MUs (C), MU twitch force (D), total number of muscle fibres (E), and muscle fibre cross-sectional area (CSA) (F) in control (CON), cross-reinnervated muscles after TIB-CP nerve-nerve (N-N) suture, nerve-muscle (N-M) suture, and nerve-nerve suture after L5 ventral root section (R-N-N) sutures.

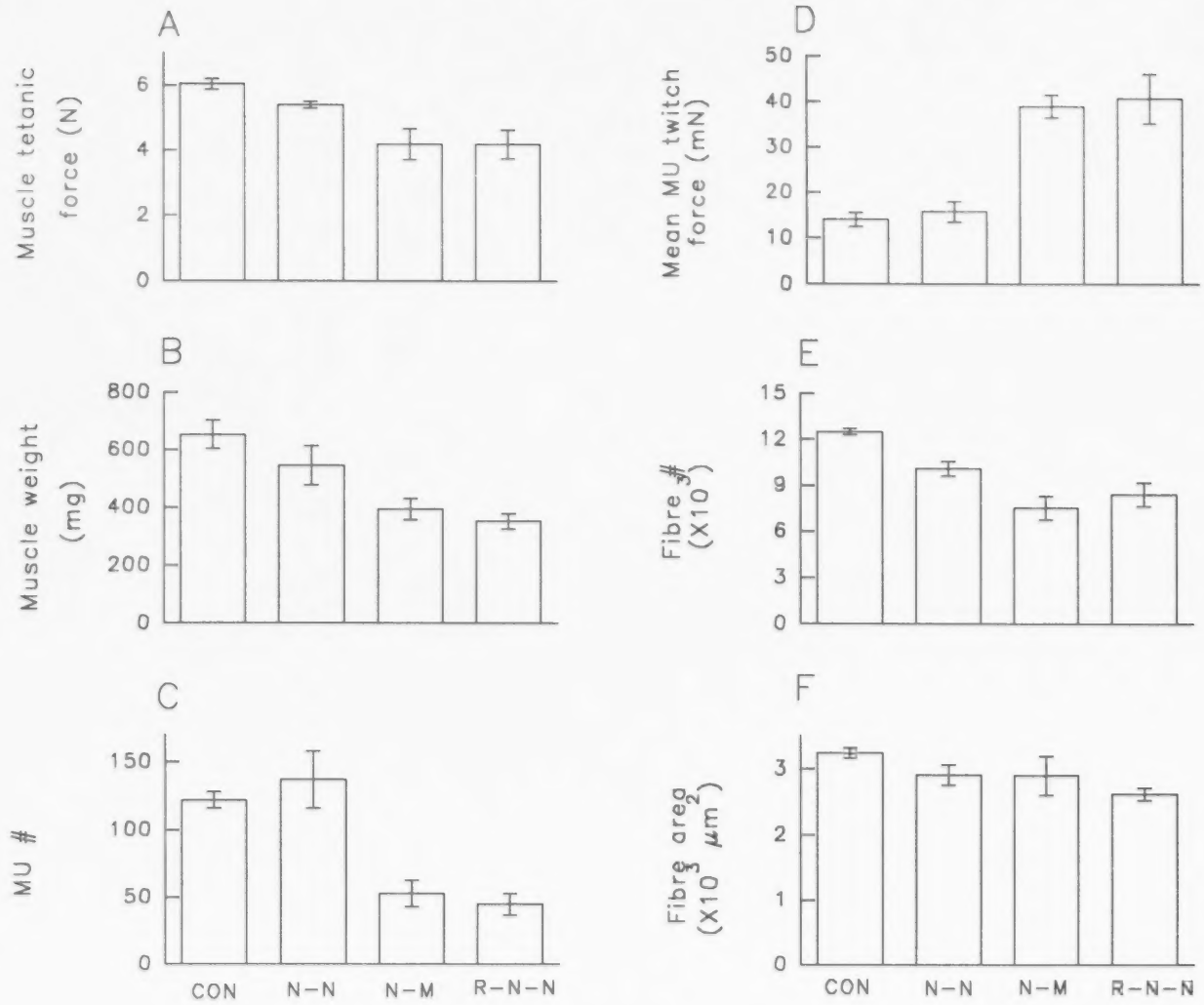

A
Muscle tetanic force (N)
0
2
4
6
B
Muscle weight (mg)
0
200
400
600
800
C
MU #
0
50
100
150
CON
N-N
N-M
R-N-N
D
Mean MU twitch force (mN)
0
10
20
30
40
50
E
Fibre # (X10)
0
3
6
9
12
15
F
Fibre area (X10 μm)
0
1
2
3
CON
N-N
N-M
R-N-N

Fig. 2.5

Cumulative distribution of motor unit twitch forces in 5 cross-reinnervated muscles after N-N suture in which the number of MUs was $53 \pm 1\%$ of normal (A) and distributions in 5 muscles after N-M suture in which the number of MUs was $55 \pm 7\%$ of the control (B). Rank order analysis, using the Kruskal-Wallis test, showed that the 5 distributions plotted in either (A) or (B) were not different from each other ($p > 0.1$). The distributions in (A) were also not different from those in (B) ($p > 0.1$).

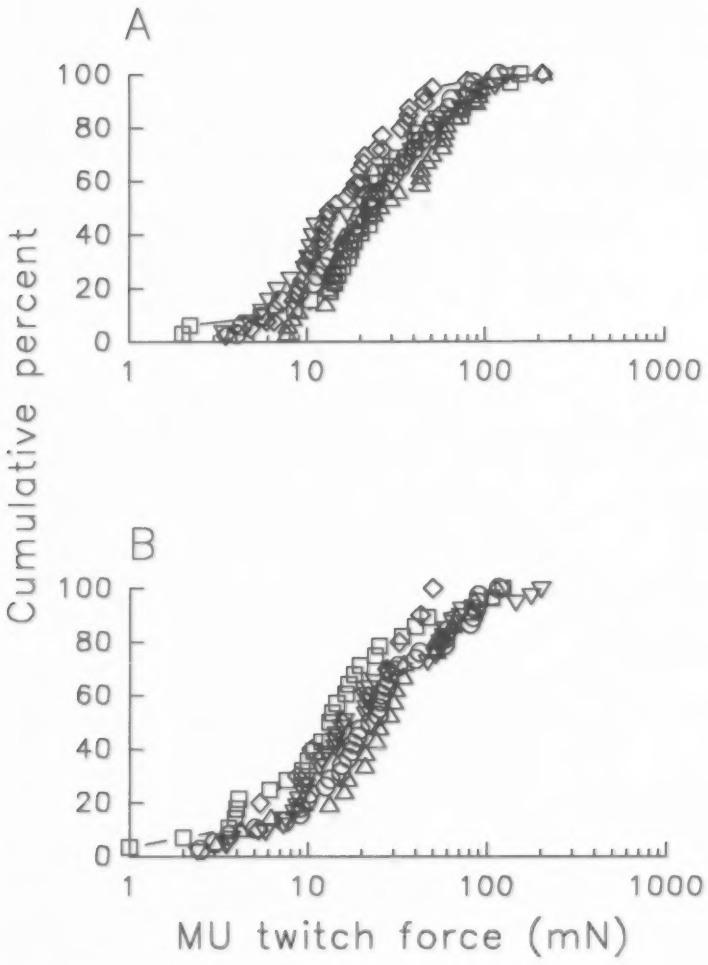

A
B
100
80
60
40
20
0
1
10
100
1000
Cumulative percent
MU twitch force (mN)

Fig. 2.6

Frequency and cumulative frequency histograms of motor unit twitch forces in control TA muscle (open bars), cross-reinnervated TA muscle after N-N (A,B, cross-hatched bars and filled circles) and N-M sutures (C,D, hatched bars, filled triangles). Distributions were shifted to the right in cross-reinnervated muscles by a factor of 2 when there was an average of 53±1% (n=5) and 56±7% (n=5) MUs of the control after N-N (A,B) and N-M (C,D) sutures respectively (mean MU force were 29.2±2.9 mN; 31.8±2.3 mN as compared to 13.9±1.5 mN in control). For an average of 18±5% MUs of the control, the MU forces increased by a factor of 4 after both N-N suture (62.7±7.1 mN) (E,F) and N-M suture (55.2±3.2 mN) (G,H). The parallel shift of cumulative histograms on semi-logarithmic scales showed that the size of all MUs increased by the same factor. Distributions and means from reinnervated muscles were different from control ($p < 0.01$). The distributions and means after N-N suture were not significantly different from those after N-M suture when there was similar number of motor units in the reinnervated muscle ($p > 0.05$).

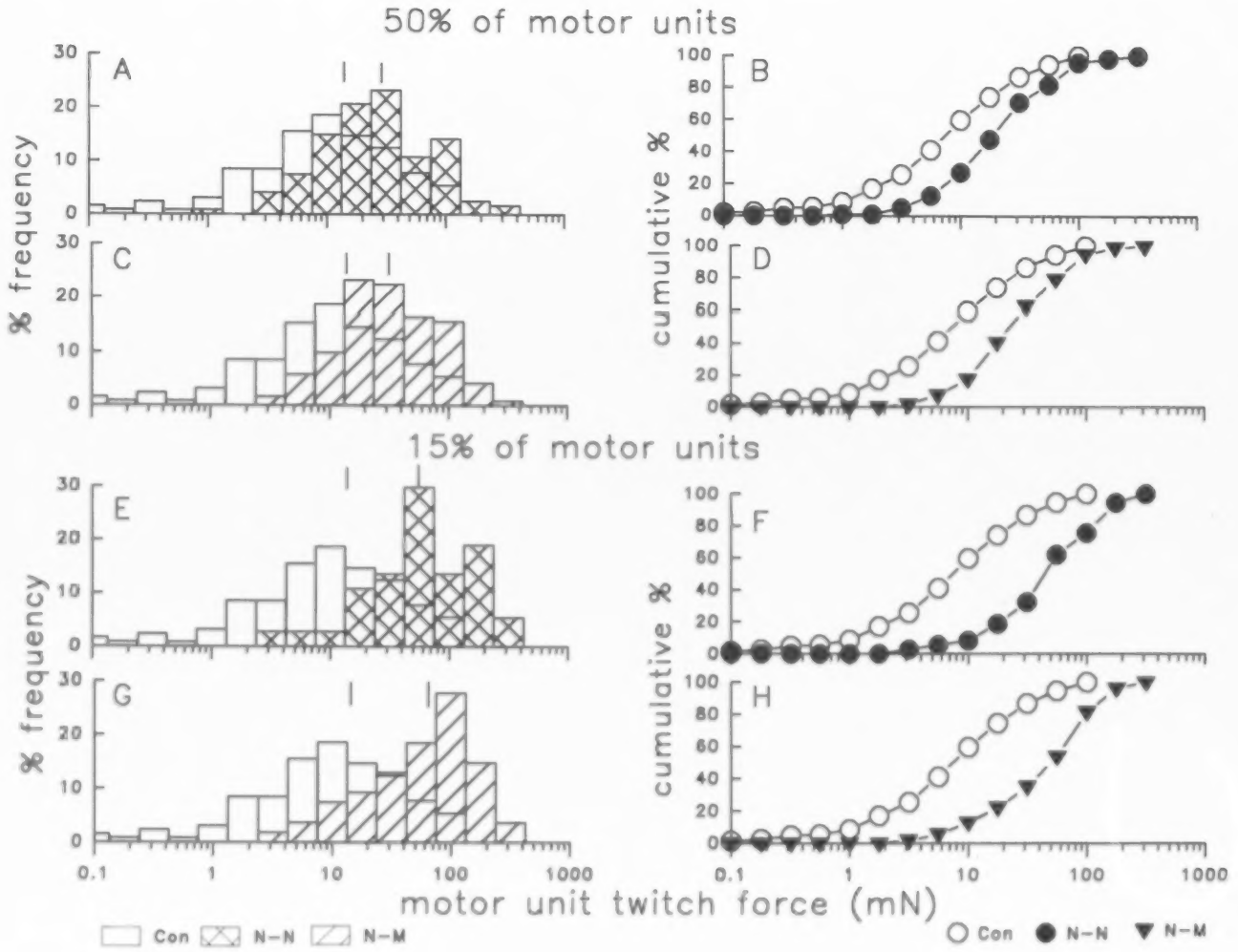

50% of motor units
15% of motor units
A
B
C
D
E
F
G
H
% frequency
cumulative %
motor unit twitch force (mN)
Con
N-N
N-M
0.1
1
10
100
1000

Fig. 2.7

Mean MU twitch forces in cross-reinnervated muscles after N-N (including R-N-N) suture (A) and N-M suture (B) plotted against the number of MUs relative to normal on double logarithmic axes. The slopes (±S.E.) of the regression lines drawn through the data points in (A) and (B) (-0.57±0.07 and -0.70±0.20) were significantly different from zero but not significantly different from each other. Thus, MUs can enlarge to the same extent after both N-N and N-M sutures.

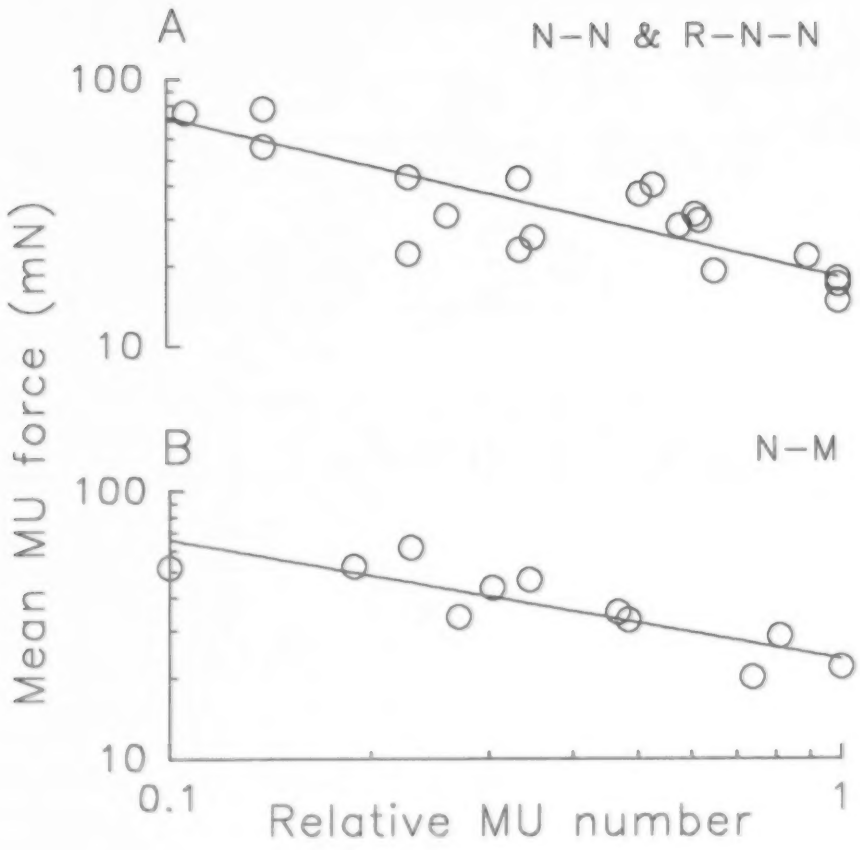

A
N–N & R–N–N
100
10
B
N–M
100
10
0.1
1
Mean MU force (mN)
Relative MU number

Fig. 2.8

Reinnervated muscle force relative to control muscle force after N-N (including R-N-N) (A) and N-M suture (B) were plotted as a function of motor unit number relative to control on a double logarithmic axes. The slopes ($\pm$S.E.) of the regression lines fitted in the data points in plot (A) (0.43 ± 0.07) and (B) (0.59 ± 0.1) were significantly different from zero, but not significantly different from each other.

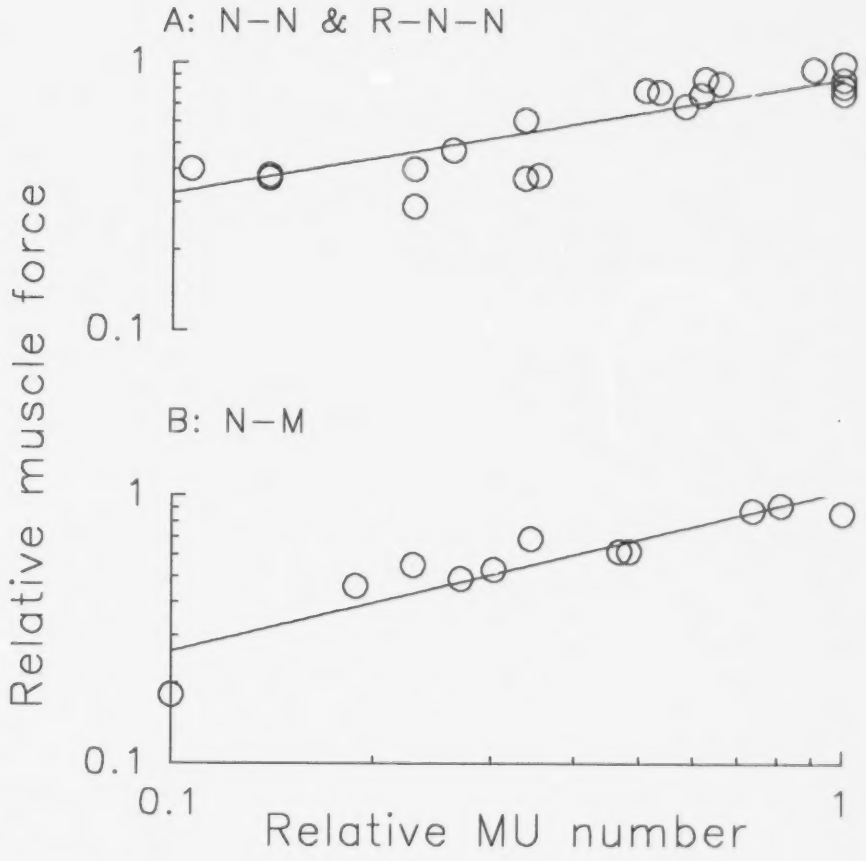

A: N–N & R–N–N
1
0.1
B: N–M
1
0.1
0.1
1
Relative muscle force
Relative MU number

Fig. 2.9

The number of muscle fibres (IR), mean fibre cross-sectional area (CSA), and specific force (SF) in single glycogen depleted MUs in reinnervated muscle after N-N (open circles) and N-M suture (filled circles) plotted as a function of MU twitch force (A,C,E) and tetanic force (B,D,F) on double logarithmic scales. The slopes ($\pm$S.E.) of regression lines for the relationships between IR and MU twitch force and that between IR and tetanic force were 0.45 ± 0.12 and 0.58 ± 0.12 respectively which were significantly different from zero, but not different from each other. The slopes ($\pm$ S.E.) of the regression lines for the relationships between fibre CSA and twitch force (0.07 ± 0.06), CSA and tetanic force (0.07 ± 0.06), SF and twitch force (0.32 ± 0.12), and SF and tetanic force (0.33 ± 0.13) were not significantly different from zero. Thus, IR is the major determinant of MU force in reinnervated muscles. The same correlations observed for IR with twitch force and IR with tetanic force showed that twitch force was as good as tetanic force to reflect the number of muscle fibres in the MU.

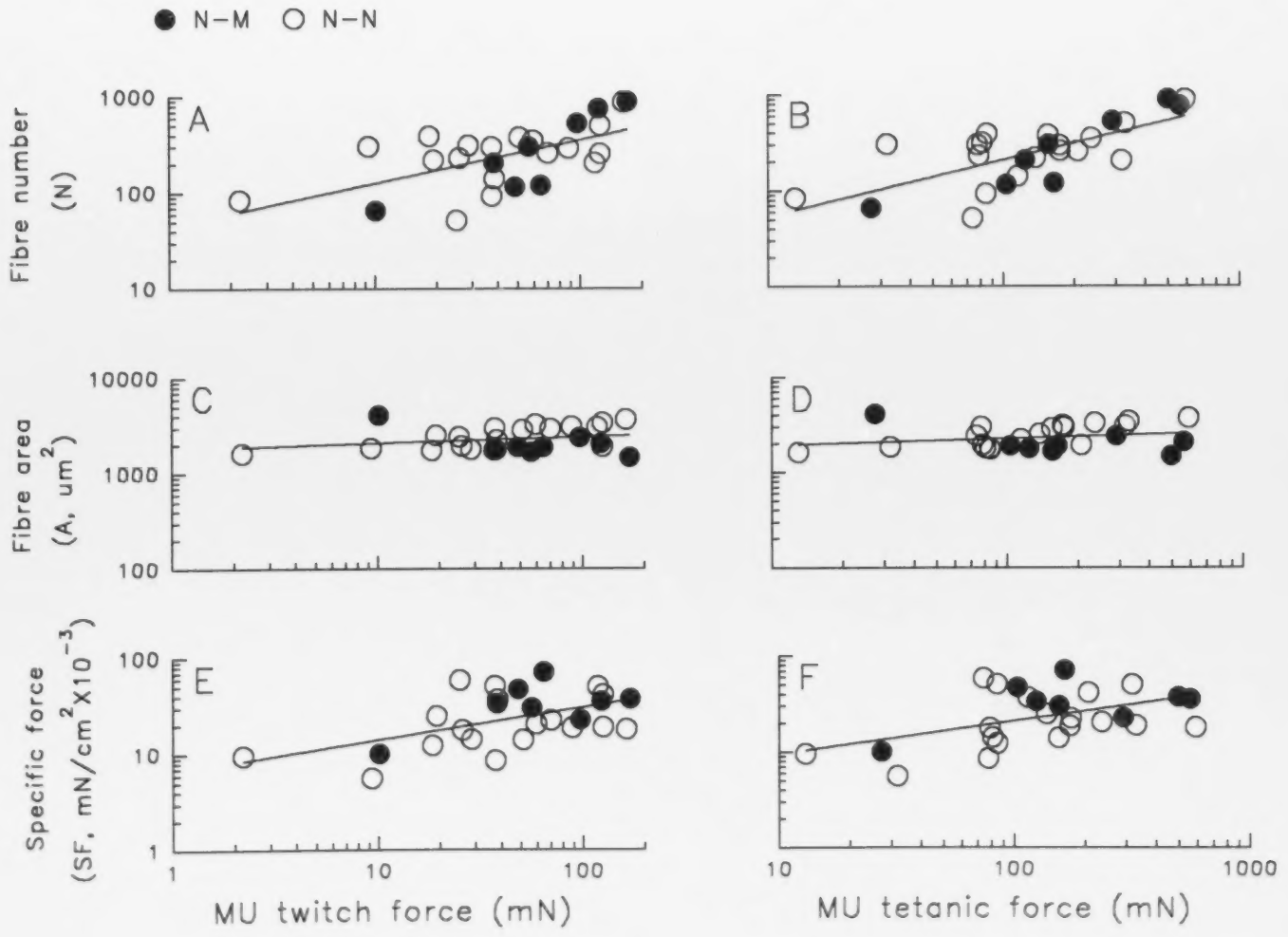

● N–M ○ N–N
Fibre number (N)
1000
100
10
A
B
Fibre area (A, um²)
10000
1000
100
C
D
Specific force (SF, mN/cm²X10⁻³)
100
10
1
E
F
1
10
100
10
100
1000
MU twitch force (mN)
MU tetanic force (mN)

Fig. 2.10

Comparison of mean MU twitch force (A), MU number (B), mean number of muscle fibres per motoneurone obtained by either dividing the total number of muscle fibres by number of MU (innervation ratio, IR) (C) or from one glycogen depleted motor unit (number of muscle fibres in each MU, N) (D) in each reinnervated muscle after N-M or R-N-N suture. Data for different reinnervated muscles were shown as open circles. Note the high variability in the parameters in both reinnervated muscles after N-M and R-N-N sutures. Mean values for all parameters in reinnervated muscles after N-M sutures were not significantly different from those after R-N-N suture ($p > 0.05$).

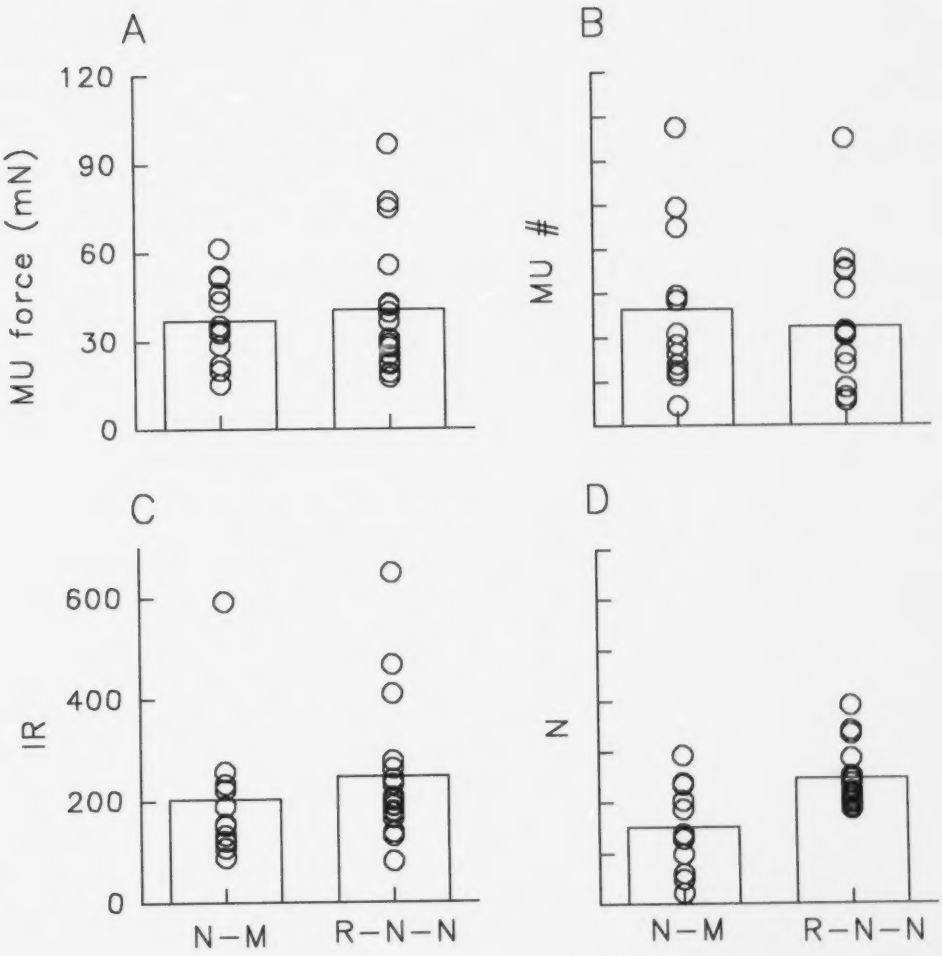
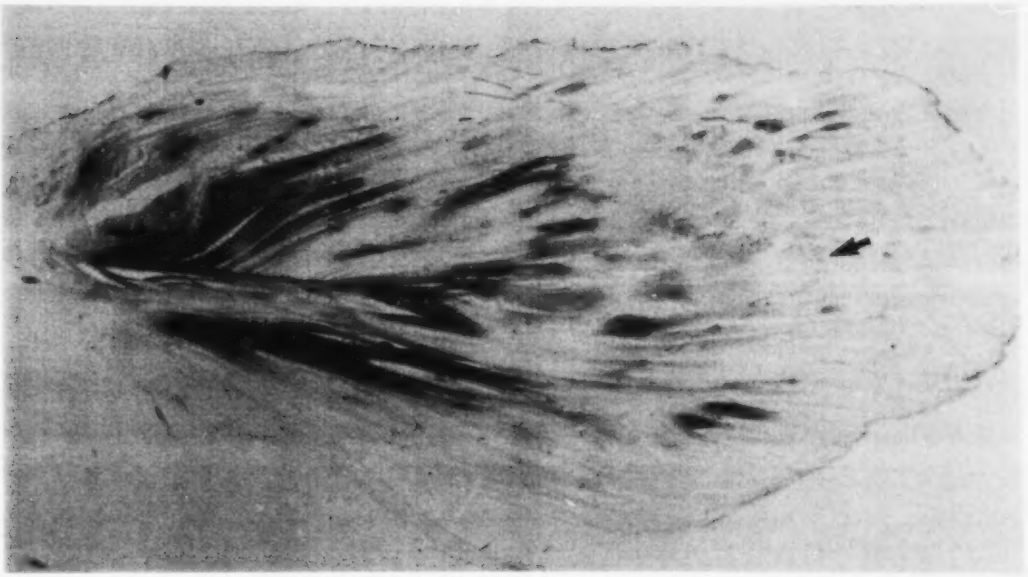

A
B
C
D
MU force (mN)
120
90
60
30
0
MU #
IR
600
400
200
0
N
N–M
R–N–N
N–M
R–N–N

Fig. 2.11

Motor end-plates and nerve fibres visualized with cholinesterase and $AgCl_2$ staining in longitudinal section of control (A) and reinnervated (B) TA muscles after N-M suture. The endplates in reinnervated muscles were distributed in a V shape (arrow), a pattern similar to the control indicating that regenerating axons had reinnervated the original endplates. Note the position of the point of TIB nerve insertion which was close to the most distal endplates and 8 mm from the most proximal endplates. The normal entry point of the CP nerve is at the level of the most proximal endplates.

Control



N–M suture

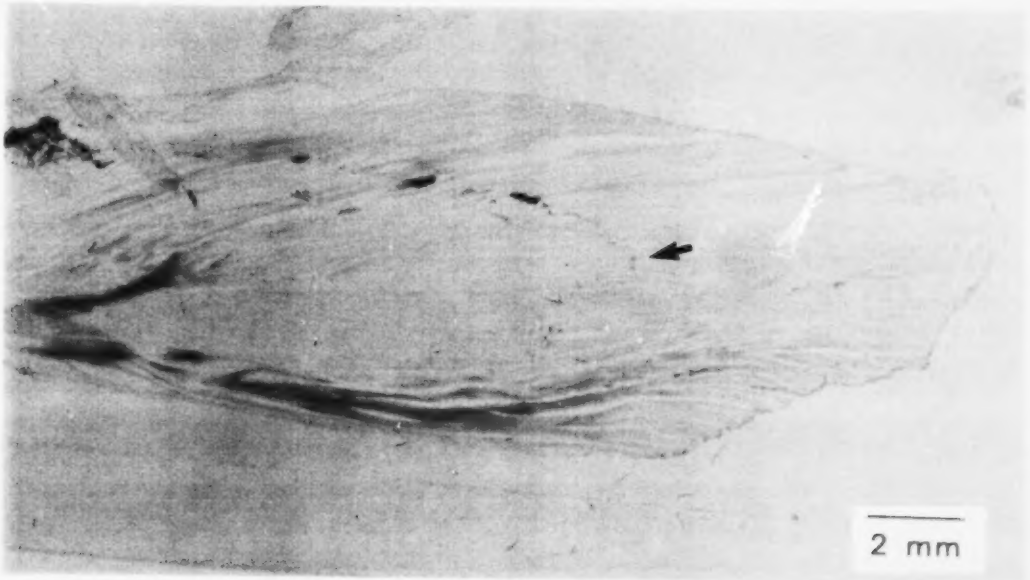
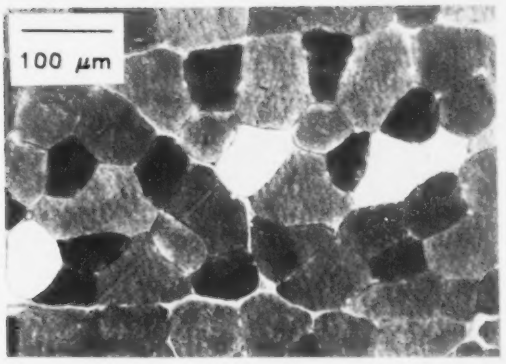
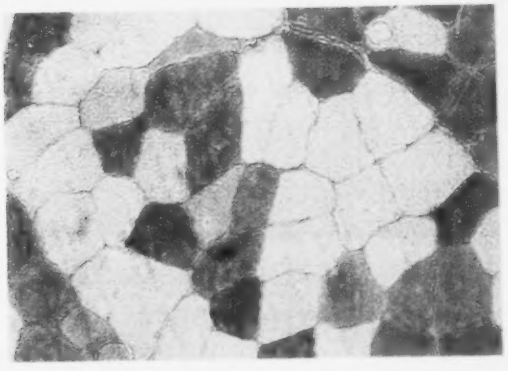


Fig. 2.12

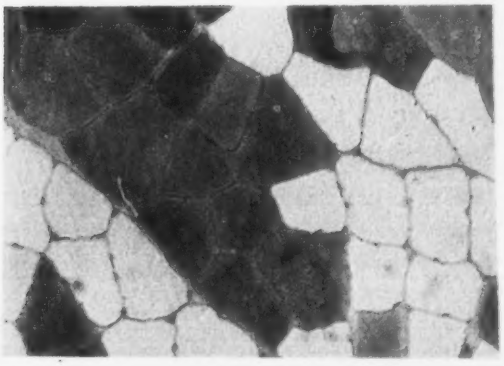
Camera lucida drawings of single MUs in muscle cross sections and photomicrographs of a selected region of a glycogen-depleted MU in control (A), and cross reinnervated muscle after N-N suture (B), N-M suture (C), and N-N suture after section of ventral root L5 (R-N-N) (D). Normally muscle fibres of a single MU are scattered and occupy a distinct territory (A). After N-N suture, the MU territory was smaller and majority of muscle fibres clumped (B). A similar spatial distribution pattern was found after N-M and R-N-N sutures (C,D). The FI MU in the deep region (A) contained 85 muscle fibres; the FI MU in the superficial medial region (B) contained 296 fibres; FI MU in the central superficial region (C) contained 48 fibres; FR MU in deep medial region (D) included 231 fibres. Note the small fibres in the FR MU (D).



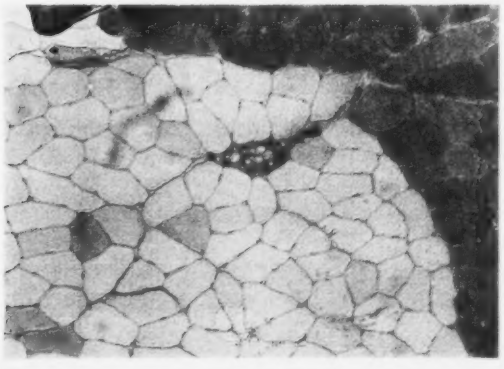
A



B



C



D

CHAPTER 3

Effects of Long-term Axotomy of Motoneurones on Nerve Regeneration and Cross-reinnervation of the Rat Tibialis Anterior Muscle

3.I...INTRODUCTION

3.II...METHODS

...1...Delayed nerve cross-anastomosis
...2...Surgical preparation for the final experiment
...3...Muscle and motor unit force recordings
...4...Muscle fibre histochemistry
...5...Data analysis
.a...MU number and size
.b...Muscle fibre number and size
...6...Statistics

3.III...RESULTS

...1...Immediate TIB-CP nerve cross-suture
...2...Prolonged TIB motoneurone axotomy
...3...Properties of the cross-reinnervated TA muscle

3.IV...DISCUSSION

...1...MU number in cross-reinnervated muscle
...2...Compensatory increase in motor unit size
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3.I...INTRODUCTION

Nerve regeneration and recovery of target function in humans are often disappointing even after microsurgical repair of injured nerves (Sunderland, 1978; Terzis & Smith, 1990). In particular, regenerative outcome may be dismal when reinnervation of denervated target organs is delayed due either to a long distance between target and lesion site or to delayed nerve repair following major trauma (Sunderland, 1978; 1991; Merle, Amend, Cour, Foucher & Michon, 1986; Birch & Raji, 1991).

The causes for poor functional recovery after delayed nerve repair are not well understood. Three factors must be considered for recovery. First, neurons must survive and synthesize material for axonal regeneration (Grafstein & McQuarrie, 1978). Second, the distal nerve stump and its associated non-neural cell population must provide adequate trophic and substrate support for nerve regeneration (Cajal, 1928; Holmes & Young, 1942). Third, regenerating axons must make functional connections with appropriate peripheral target(s) and the target organ must fully recover from denervation atrophy.

Unlike dorsal root ganglion neurons and cranial motoneurones (Aldskogius & Arvidsson, 1978; Arvidsson & Aldskogius, 1982; Himes & Tessler, 1989), axotomized spinal motoneurones in adults survive for long periods of time (Carlsson, Lais & Dyck, 1979; Melville, Sherburn & Coggeshall, 1989; Gordon *et al.*, 1991). The capacity of injured nerves to regenerate and the ability of denervated muscles to accept reinnervation after delayed nerve repair were first addressed half a century ago (Holmes & Young,

1942; Gutmann & Young, 1944). In order to separately prolong axotomy and denervation, they used a cross nerve anastomosis regime in the rabbit hindlimb. Holmes & Young (1942) reported that the number of axonal sprouts in the distal nerve stump was the same one month after suturing the long-term axotomized tibial nerve to a freshly cut common peroneal nerve as after immediate nerve repair. Since the rate of sensory nerve regeneration was also the same, the authors concluded that long-term axotomy did not compromise nerve regeneration. Because Gutmann & Young (1944) also observed a progressive failure of muscle reinnervation after prolonged muscle denervation, poor functional recovery after delayed nerve repair has been attributed to the inability of denervated muscle to accept nerve reinnervation and to recover from denervation atrophy (see also Bowden & Gutmann, 1944). However, it is still unclear as to whether long-term axotomy is detrimental to functional recovery.

Although regenerating axons have been shown to enter the distal nerve stump (Holmes & Young, 1942), it is possible that after long-term axotomy these may fail to elongate over the distance required to reach denervated muscle and/or to make functional nerve-muscle connections. To clarify the effect of prolonged axotomy, we have used 1) the same nerve cross-anastomosis paradigm described previously to prolong motoneurone axotomy prior to nerve repair (independent of muscle denervation) and 2) electrophysiological and morphological methods to quantify the number of regenerating motor axons which successfully reinnervated freshly denervated muscles, the number of muscle fibres reinnervated by each axon and the capacity of long-term axotomized axons

to reverse denervation atrophy. Our results show that long-term axotomy does reduce the number of motor axons which successfully reinnervate muscle but not the number of muscle fibres reinnervated by each motor axon. Some of these results have been described in abstract form (Fu & Gordon, 1993*a, b*).

3.II...METHODS

3.II.1...Delayed nerve cross-anastomosis.

Under general anaesthesia induced by intra-peritoneal (*i.p.*) injection of sodium pentobarbital (45 mg/kg) and aseptic conditions, the posterior tibial (TIB) nerve on the right side was transected near the ankle in 32 female Sprague-Dawley rats (body weight: 150-200 g). The site of section was distal to the exits of branches to the triceps surae, the plantaris, the flexor hesllicis longus and the digitorum longi muscles. As shown in Fig. 3.1, the proximal stump of the cut TIB nerve was either cross-sutured immediately to the distal stump of the cut common peroneal (CP) nerve close to the knee (immediate nerve suture) or ligated and sutured to the normally innervated lateral gastrocnemius muscle to prevent nerve regeneration for up to one year (Watson, 1970). Fourteen to 365 days later, the long-term axotomized TIB stump was refreshed (by removing 5 mm from the ligation site) and sutured to the distal stump of a CP nerve which was freshly cut close to the anterior muscle compartment of the hindlimb (delayed nerve suture). The proximal stump

of the CP nerve was ligated and sutured to the normally innervated biceps femoris muscle to prevent self-reinnervation of the tibialis anterior (TA) muscle.

3.II.2...Surgical preparation for the final experiment.

Three to 17 (mean±S.E: 11.9±0.8) months after TIB-CP cross-suture, the TA muscle was isolated in a final experiment for muscle and MU force recordings. Three to 5 days prior to the final experiment, 5% glucose was added in drinking water to increase muscle glycogen storage. Atropine sulphate (0.1 mg/Kg) was injected to reduce tracheal secretion. General anaesthesia was induced by sodium pentobarbital (45 mg/Kg i.p.), monitored by continuous ECG recording and maintained by intravenous injection of 5% of the original sodium pentobarbital dose diluted 20% in 5% dextrose-saline via a cannula inserted into the right external jugular vein. The trachea was cannulated for mechanical ventilation when necessary. Blood volume was maintained by hourly injection of 0.5-1 ml of the 5% dextrose saline solution via the i.v. cannula.

All hindlimb muscles innervated by L4-L6 ventral roots were denervated except the TA (Fig. 3.2). Both TA muscles were isolated with their distal tendons attached to small pieces of bone for later attachment to force transducers. Bipolar surface patch electrodes were sewn onto the muscle fascia for either EMG recordings or direct muscle stimulation (Fig. 3.2). The bared ends of two teflon coated fine silver wires, used as stimulating electrodes, were inserted into the muscle beneath the sciatic nerve for eliciting muscle contractions. Both hindlimbs were immobilized with clamps at the knees and

ankles. The distal tendon of the TA muscle was attached to a (custom-made) force transducer for recording muscle and MU forces. Leg and rectal temperatures were maintained at 30-32°C and 35-37°C respectively with a heating pad and overhead electric bulb.

Laminectomy of T13 to L6 was performed. Dura mater was cut and ventral roots L4 to L6 were isolated with a glass rod and gentle suction using glass pipette. A mineral oil pool was prepared for the spinal cord and the exposed ventral roots by stretching the skin flaps around the incision. Stimulating electrodes for ventral roots were placed above the spinal cord in the mineral oil pool.

3.II.3...Muscle and motor unit force recordings.

Force and EMG signals were amplified, monitored on a Tektronix dual time base oscilloscope (model 5441), recorded continuously on a Gould 1200S pen recorder and digitized and stored with a LSI-11 computer.

The TA muscle was adjusted to its optimal length for recording maximum isometric twitch and tetanic forces in response to supra-threshold stimulation of the sciatic nerve. Two to 5 EMG and force responses to stimuli of 1, 5 and 21 pulses at 100 Hz were averaged on line. Fine filaments containing 3-7 axons in ventral roots L4, L5 and L6 contributing to the tibial nerve were stimulated to elicit all-or-none EMG and incremental force responses (Fig. 3.2). Three to 10 EMG and twitch force responses to 1 Hz stimulation were averaged per increment and the twitch response of each MU was obtained

by digital subtraction. Ten to 50 MUs in each reinnervated TA muscle were sampled, which represented at least 30% (and up to 100%) of the total MU population in the muscle.

One MU per muscle was isolated for extensive characterization and glycogen depletion. MUs were classified as slow (S), fatigue resistant (FR), fast fatigue intermediate (FI) and fast fatiguable (FF) on the basis of contractile speed, "sag" and fatigability (for details, see Totosy de Zepetnek, Zung, Erdebil & Gordon, 1992*a*). The criteria used for single MU isolation were an all-or-none twitch contraction and associated EMG response. A single MU was only selected for further characterization if the threshold voltage of its axon was less than 10 volts and at least 10 times lower than the thresholds for any other motor axon in the same ventral root filament. These criteria were rigidly met in order to keep the unit for the 1-3 hours of recordings and the subsequent glycogen depletion. The isolated axon for a single MU was stimulated repetitively using trains of stimuli to deplete its glycogen content. Details of the stimulation protocol were described previously (Totosy de Zepetnek, Zung, Erdebil & Gordon, 1992*b*; Chapter 2). Briefly, MUs were stimulated for up to 4 hours with as many as 10 repeated cycles of brief 50 Hz tetanic trains (5 pulses at an interval of 20 ms). Each cycle repeated in trains at 1-9 Hz until force declined to $< 20\%$ and the same tetanic trains at 0.25 Hz to allow force recovery.

3.II.4...Muscle fibre histochemistry.

TA muscles were quickly removed, weighed, cut into 3 blocks, rapidly frozen in isopentane cooled with liquid nitrogen, and stored in a freezer at -70°C for subsequent sectioning and histochemical staining. Serial cross-sections each of 10 μm thick were cut and stained for 1) glycogen with Periodic Acid Schiff (PAS) reaction according to Pearse (1960), 2) myosin ATPase following acid pre-incubation according to Green, Reichmann & Pette (1982) after pre-incubation at pH 10.4 modified from Guth and Samaha (1970) and 3) NADH according to Dubowitz and Brooke (1973). Muscle fibres were classified into slow oxidative (SO), fast oxidative glycolytic (FOG), and fast glycolytic (FG) types as previously described (Totosy de Zepetnek *et al.*, 1992b).

3.II.5...Data analysis.

MU number and size. At least 30% of the total MU population in each reinnervated muscle was sampled to obtain a representative mean of MU twitch force. The total number of MUs in each muscle was calculated by dividing the whole muscle twitch force by the mean MU twitch force. Shown as an example in Fig. 3.2, the average muscle twitch force was 1990 mN and the mean MU twitch force was 14 mN obtained from a sample of 45 MUs. The calculated MU number was therefore 140.

Muscle fibre number and size. The total number of muscle fibres in each muscle was calculated by multiplying muscle cross-sectional area (CSA) by muscle fibre density. Muscle CSA was measured on cross-sections which contained the maximum number of

muscle fibres. In the same cross-sections, muscle fibres were counted in 0.63 mm^2 areas located in the same 6-9 regions in all the muscles. The count included at least 10% of the total muscle fibre number in each muscle. Muscle fibre density was measured in the same 6-9 regions in order to take into account of regional difference in fibre size (Pullen, 1977; Parry & Wilkinson, 1991; Totosy de Zepetnek *et al.*, 1992*a*). The remaining error due to region selection was estimated by counting the number and size of muscle fibres in the same regions of both the left and right TA muscles in 3 control rats. The difference between the left and right sides was less than 10%.

The average muscle fibre CSA was obtained by dividing the total muscle CSA by the total number of muscle fibres in each muscle. In order to validate this estimate, we compared the calculated muscle CSA with the directly measured fibre CSA of 500-1000 fibres within the 6-9 regions in 4 reinnervated muscles. Good agreement was found between direct measurement and indirect estimate of mean muscle fibre CSA (2912 ± 63 μm^2 compared to 2985 ± 166 μm^2). The indirect method was therefore conventionally used to acquire the mean muscle fibre CSA in all the control and cross-reinnervated TA muscles.

3.II.6...Statistics.

Arithmetic means were calculated and shown with standard errors (mean$\pm$S.E). One-way analysis of variance (ANOVA) was applied to examine differences for all the parameters (muscle force, MU number and force, muscle and muscle fibre number and

CSA) between control, cross-reinnervated muscles after immediate nerve repair and muscles which were cross-reinnervated after varying the duration of axotomy. Bonfferoni tests were used to detect any differences between all the possible combinations of paired conditions. The Kruskal-Willis test of rank order was used to examine the differences in the distribution of muscle fibre CSA between control and cross-reinnervated muscles. Regression lines were fitted using least square analysis. For all the above statistical analyses, p values of < 0.05 were regarded as significant.

3.III...RESULTS

A total of 64 female rats were used: 32 were unoperated controls and 32 underwent nerve cross-suture surgery in which TIB nerve was cut and sutured to the distal stump of the freshly cut CP nerve (N=6) either immediately or after a delay of 14 to 365 days (N=26). Control muscle forces were recorded in normal TA muscles from age-matched unoperated rats (N=32; body weight: 291 $\pm$ 70 g). The final body weight of the operated rats was 344 $\pm$ 9 g (range: 260-490 g).

Cross-reinnervated TA muscle and MU forces were recorded 3 to 17 months after TIB-CP cross reinnervation because recovery of reinnervated TA muscles is stable by 3 months after nerve-nerve suture near the entry of the CP nerve to the anterior muscle compartment of the hindlimb (Totosy de Zepetnek *et al.*, 1992*a*). Muscle and MU forces were compared with unoperated control TA muscles rather than contralateral TA muscles

which showed a concurrent compensatory hypertrophy with increasing duration of axotomy (Fig. 3.3).

3.III.1...Immediate TIB-CP nerve cross-suture.

Reinnervated TA muscles developed smaller tetanic forces than the control (Fig. 3.4A) in parallel with a small reduction in muscle weight (545 ± 68 g as compared to 627 ± 154 g for the control; $p < 0.05$). Twitch forces developed by the reinnervated muscles (Fig. 3.4B) and their MUs (Fig. 3.4C) were not different from the control ($p > 0.05$). When muscle twitch force was divided by MU twitch force to obtain the MU number, it was evident that as many TIB motor axons reinnervated the TA muscle as the CP nerve normally does (Fig. 3.4D). Since the mean muscle fibre CSA was also the same as the control (Fig. 3.4E), the small but significant reduction in the tetanic force of reinnervated muscles is due to a decrease in the number of muscle fibres (Fig. 3.4F).

3.III.2...Prolonged TIB motoneurone axotomy.

When TIB motoneurones were axotomized for up to one year prior to TIB-CP cross-suture, the reinnervated TA muscles recovered as well as after immediate nerve cross suture. This was apparent from the size of the muscle (Fig 3.5) and CSA of its muscle fibres (Fig. 3.5 & 3.6) and mean isometric force (Fig. 3.7). Although there was some variation between rats, muscle force did not decline with prolonged axotomy prior to nerve suture (Fig. 3.7A). This applied to both twitch and tetanic forces. However, the

number of reinnervated MUs declined exponentially (τ = 40 days) to less than 35% of the number in reinnervated muscles after immediate nerve suture (Fig. 7B).

MU enlargement compensated for the reduction in MU number. MU force increased 2 to 3 times (Fig. 3.7C) and was accompanied by a similar increase in the number of muscle fibres per motoneurone (the innervation ratio, IR; Fig. 3.7D). The enlargement of MUs was sufficient to reinnervate the available denervated muscle fibres so that the number of reinnervated muscle fibres was the same as after immediate nerve suture (Fig. 3.7E). Prolonged axotomy therefore reduced the ability of motor axons to regenerate and/or to make functional connections but not their ability to branch and to reinnervate denervated muscle fibres. In addition, prolonged axotomy did not reduce the capacity of regenerating nerve fibres to reverse denervation atrophy in reinnervated muscles (Fig. 3.7F).

The effects of prolonged axotomy are summarized in Fig. 3.8. Significantly fewer axons reinnervated denervated TA muscles but each axon reinnervated 3 times as many muscle fibres as after immediate nerve repair. As a result, as many muscle fibres were reinnervated despite the 3-fold reduction in MU number as compared with after immediate nerve suture. Since all reinnervated muscle fibres recovered their normal size, the muscles developed as much force as after immediate nerve suture.

3.III.3...Properties of the cross-reinnervated TA muscle.

Contractile speed of the cross-reinnervated TA muscles was not affected by prolonged axotomy even though there was a small but significant increase in the proportion of slow muscle fibres in muscles reinnervated after 1 year of axotomy (Fig. 3.9).

As shown in Fig. 3.10, twitch and tetanic forces correlated well with the number of muscle fibres reinnervated by each motoneurone (innervation ratio, IR), and to a much lesser degree with mean MU fibre CSA and specific force. The slope of the regression line between force and IR was steeper than in normal because MU force depends more on the N than on the mean CSA and specific force after cross-reinnervation as found previously in self-reinnervated muscles (Totosy de Zepetnek *et al.*, 1992*a*). Furthermore, the correlation between twitch force and IR is the same as that for tetanic force vs IR showing that the twitch force is as good an indicator as tetanic force for MU size in terms of the number of muscle fibres reinnervated per motoneurone.

3.IV...DISCUSSION

The present results demonstrate that prolonged axotomy significantly reduces the number of motor axons which successfully regenerate and make functional connections with denervated muscle fibres. Thus, long-term axotomy is an important contributing factor to poor functional recovery observed in animal models and presumably human

patients when nerve repair is delayed. Nevertheless, the normal capacity of the neurons to form enlarged MUs may compensate for the reduction in the number of MUs showing that long-term axotomy does not compromise the number of muscle fibres reinnervated per axon.

3.IV.1...MU number in the cross-reinnervated TA muscle.

An average of 137 TIB motoneurones made functional connections with denervated TA muscles after immediate TIB-CP cross suture. This is similar to the number of CP motoneurones which normally supply the TA muscle (Fig. 3.4). This number, calculated from the ratio of muscle force and average MU force (McComas, 1991), is about a third of the 500-600 motoneurones which send their axons to the intrinsic muscles of the foot via the posterior TIB nerve and to the dorsiflexor muscles via the CP nerve (Crockett, Harris & Egger, 1987; Swett, Wikholm, Blanks, Swett & Conley, 1986) and represents a large proportion of the 300-400 α-motoneurones in the motor pools (Boyd & Davey, 1968). Considering that the regenerating nerves supply several flexor muscles in addition to the TA muscle, the TIB motor axons were very successful in reinnervating muscles after immediate nerve suture. In contrast, an average of 50 TIB motoneurones made functional connections when axotomy was prolonged (6-12 months) prior to nerve repair.

After prolonged axotomy, axons either regenerated but did not succeed in reinnervating denervated muscle fibres or failed to grow over the distance and reach the denervated muscles. The former explanation is unlikely because the axons which

succeeded in forming functional MUs reinnervated many muscle fibres. It is more likely that many regenerating axons failed to elongate over the distance required to reach the denervated muscle fibres.

The conclusion that prolonged axotomy reduces the capacity of motoneurones to regenerate is in apparent contrast to the conclusions of Holmes & Young (1942) in their study where rabbit tibial nerve was axotomized and later cross-sutured to the distal stump of the sectioned CP nerve at the level of thigh rather than the ankle. These authors reported that the tibial nerve which was cut 3-365 days prior to cross-suture sent as many regenerative sprouts into the freshly cut CP distal nerve stump as after immediate cross-nerve suture. Axonal counts in the distal CP nerve stump 15-25 days after cross-suture were similar to those after immediate TIB-CP cross-suture. Furthermore, the rate of sensory nerve regeneration was not affected by prolonged axotomy. They therefore concluded that the regenerative capacity of the central nerve stump does not diminish "during the progressive formation of a neuroma".

Nonetheless, our findings are not incomparable with those of Holmes and Young (1942) but argue against their conclusions. Counts of regenerating axons in the distal nerve stump within 2 weeks of TIB-CP suture reflect the early outgrowth of sprouts from the proximal stump of long-term axotomized sensory and motor axons in which each parent axon may give rise to as many as 20 daughter sprouts (Aitken, Sharman & Young, 1947; Toft, Fugleholm & Schmalbruch, 1988; Mackinnon, Dellon, and O'Brien, 1991). Most sprouts are withdrawn when regenerating axons make functional target connections

to leave one regenerated sprout per parent axon (Aitken *et al.*, 1947; Toft *et al.*, 1988). However the withdrawal may take many months (MacKinnon *et al.*, 1991). Before connections are made, axonal counts greatly over-estimate the number of nerve fibres which successfully regenerate over distance to make functional target connections. For motoneurones, physiological methods to count MU provide a useful means to quantify the number of axons which regenerate successfully and form functional nerve-muscle connections. The MU estimates in the present study show convincingly that the number was significantly reduced after prolonged axotomy. Retrograde labelling of motoneurones in reinnervated muscles is another method for counting axons which reach the muscle but the counts will inevitably include those axons which reach the muscle but do not form functional connections. In addition, accurate labelling is highly dependent on good penetration of the label (Swett *et al.*, 1986).

The number of regenerating axons which make functional connections declines as a function of the duration of axotomy prior to nerve suture. The decline was exponential with a time constant of 40 days. The asymptotic level corresponds to 35% of the number after immediate nerve suture (Fig. 3.7). It is probable that the capacity of axotomized motoneurones to regenerate their axons progressively declines with prolongation of axotomy prior to refreshing the nerve for delayed nerve repair. Growth-associated events in axotomized motoneurones which include upregulation of tubulin and actin may not be optimally retained when regeneration is temporarily prevented (Cassar & Tetzlaff, 1991). Furthermore, growth attempts may be dissipated by formation of neuroma and subsequent

axonal growth within it. Interestingly, long-term axotomized motoneurones show the same qualitative response to a second axotomy normally by upregulation of tubulin and GAP-43 mRNA, downregulation of neurofilament protein (Cassar & Tetzlaff, 1991) and expression of the T-α1 tubulin isoform normally associated with neuronal growth (Cassar, Lindsay, Magi, Birdsell & Tetzlaff, 1991). However this neuronal growth response may not be sustained long enough to support elongation even in the permissive growth environment in freshly cut distal nerve stump. In addition, proliferation and migration of Schwann cells in and from the proximal nerve stump may decline with time resulting in less effective union of the proximal and distal nerve ends (Holmes & Young, 1942).

The exponential fall in the number of regenerating axons after prolonged axotomy follows the same time course as the reduction in axonal calibre (Gordon, Gillespie, Orozco & Davis, 1991). It is not clear whether this coincidence is functionally important but also consistent with the idea that axonal atrophy is linked to a decline in regenerative capacity. Findings that some nerves regenerate successfully and others do not may possibly be linked to differences in the size of motoneurones and their axons which are preserved after axotomy despite atrophy (Gordon & Stein, 1982).

3.IV.2...Compensatory increase in motor unit size.

The increase in both MU force and the number of muscle fibres per motoneurone (IR) clearly indicates that the branching ability of regenerating nerve fibres is not compromised by prolonged axotomy. An average of 3 fold, with a maximum of 5 fold,

increase in the number of muscle fibres reinnervated per motoneurone is the same as that of uninjured nerves during sprouting in partially denervated muscles (Brown & Ironton, 1978; Rafuse *et al.*, 1992). The regenerating nerves which successfully elongate to reach the denervated muscles appear to respond to local factors in the intramuscular nerve sheaths and denervated muscle fibres which promote nerve branching and synapse formation. These include the intrinsic cell adhesion molecules which are expressed by Schwann cells within the intramuscular sheaths and denervated muscle membranes and the extrinsic molecules of the extracellular basal lamina (Schachner, Antonicek, Fahrig, Faissner, Fischer, Kunemund, Martini, Meyer, Persohn, Pollerberg, Probstmeier, Sadoul, Sadoul, Seilheimer & Thor, 1990; Sanes & Hall, 1993; Martini, 1994).

An average of 3-fold increase in MU size largely compensates for the reduction in the number of reinnervated MUs. As a result, long-term axotomized TIB axons reinnervated as many muscle fibres as freshly cut TIB nerve does and the reinnervated TA muscles produced as much force as those reinnervated by freshly cut nerves.

3.IV.3...Properties of cross-reinnervated TA muscle.

After cross reinnervation by the TIB nerve, contraction speed of the TA muscle was the same as the control. The duration of axotomy did not have any significant effect on time to peak twitch force in spite of a small increase in the proportion of slow fibres after one year of TIB axotomy prior to cross-nerve suture (Fig. 3.9). This small increase in the number of slow fibres cannot be detected by the time to peak twitch force of the

whole muscle since time to peak twitch force is normally dominated by the fast muscle fibres (Gillespie, Gordon & Murphy, 1986).

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Fig. 3.1

Diagrammatic illustration of neural innervation of TA muscle, tibial nerve axotomy, and TIB-CP cross-suture. A: The sciatic nerve normally branches into the common peroneal (CP) nerve and posterior tibial (TIB) nerve. The CP nerve innervates muscles in the anterior compartment of the hindlimb including the tibialis anterior (TA) muscle (darkened). The TIB nerve innervates the intrinsic muscles of the foot (not shown). B: The TIB nerve was cut and its proximal stump ligated and sutured to the innervated triceps surae muscle to prevent regeneration for 0 to 12 months. C: Up to one year later, the CP nerve was cut and its proximal stump ligated and sutured to biceps femoris muscle to prevent self-reinnervation of TA muscle. In the same operation, the proximal TIB stump was refreshed and sutured to the freshly cut distal CP stump to promote TIB nerve regeneration and cross-reinnervation of TA muscle.

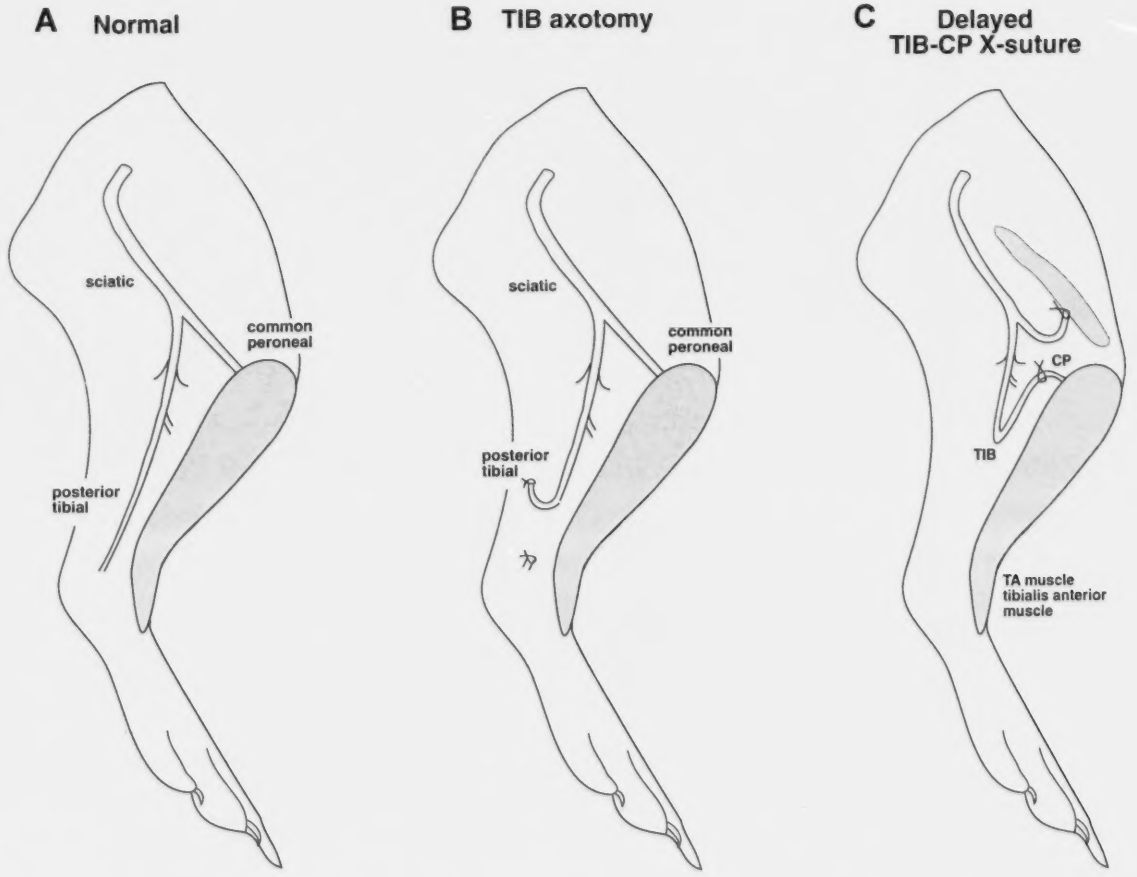

A Normal
sciatic
common peroneal
posterior tibial
B TIB axotomy
sciatic
common peroneal
posterior tibial
C Delayed TIB-CP X-suture
CP
TIB
TA muscle tibialis anterior muscle

Fig. 3.2

Muscle and MU twitch force recordings and the method used to calculate the number of MUs. A: Tibialis anterior (TA) muscle was isolated by denervating all other hindlimb muscles and attached to a force transducer. Ventral roots L5-L6 were isolated and split for stimulation of single motor axons contributing to TA muscle. B: Twitch contractions of the whole muscle and single MUs were elicited by maximal nerve stimulation and ventral root filament stimulation respectively. Filaments were teased so that stimulation elicited up to 7 all-or-none increments of force in the TA muscle. Individual MU twitch contractions were obtained by digital substraction (for details see text). C: The number of MUs for each muscle was calculated by dividing twitch force of the muscle by mean MU twitch force (averaged in Fig. 3.2B). In this example, the average muscle twitch force is 1990 mN and the mean MU twitch force is 14 mN (n=45). The calculated MU number was therefore 140.

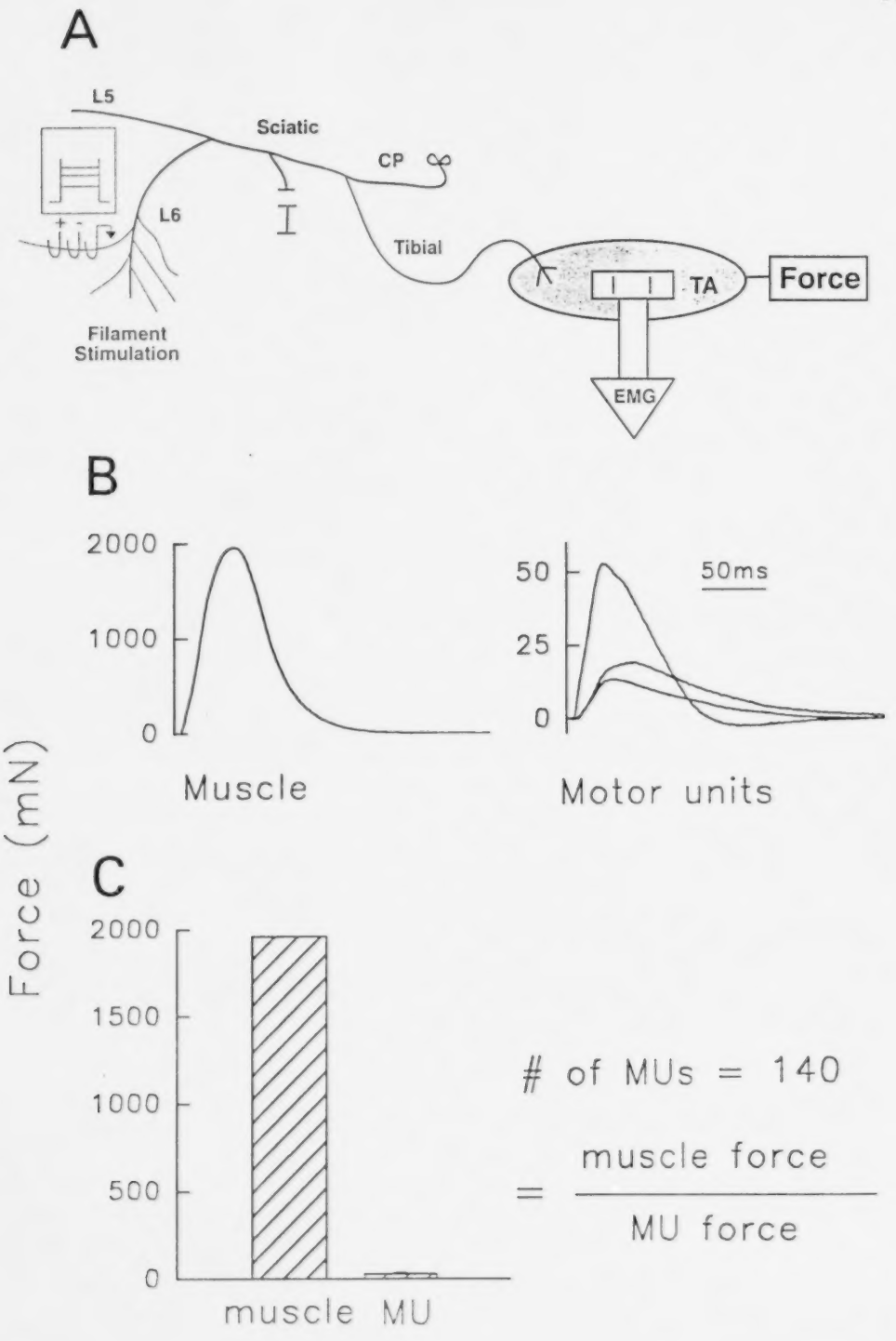

A
L5
Sciatic
CP
L6
Tibial
TA
Force
Filament
Stimulation
EMG
B
2000
1000
0
50
25
0
50ms
Muscle
Motor units
Force (mN)
C
2000
1500
1000
500
0
muscle MU
# of MUs = 140
= muscle force / MU force

Fig. 3.3

Mean (±S.E.) twitch (A) and tetanic (B) forces of reinnervated (filled circles) and contralateral (open circles) TA muscles plotted against the duration of axotomy prior to TIB-CP cross-suture. When TIB-CP cross suture was delayed for longer than 30 days, the unoperated contralateral TA muscles developed significantly larger forces than the cross-reinnervated muscles. This compensatory hypertrophy is not seen after immediate nerve repair.

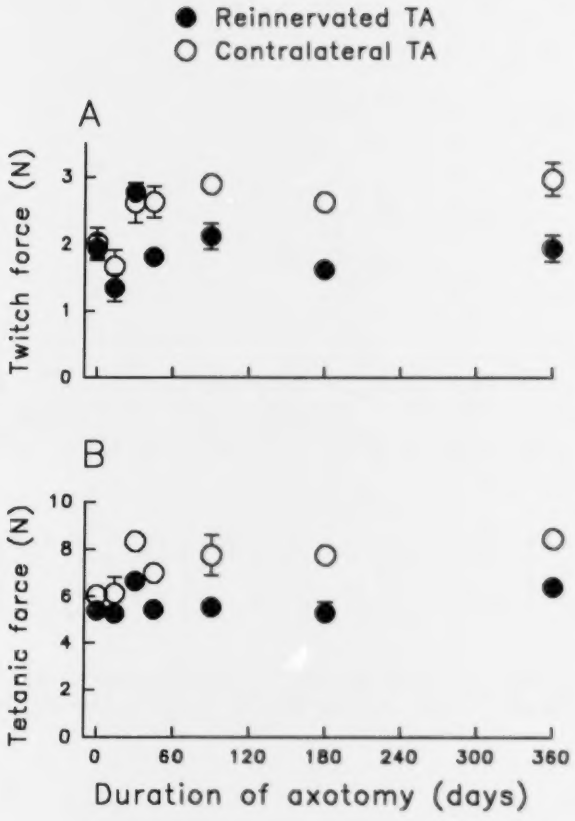

Reinnervated TA
Contralateral TA
A
Twitch force (N)
0
1
2
3
B
Tetanic force (N)
0
2
4
6
8
10
0
60
120
180
240
300
360
Duration of axotomy (days)

Fig. 3.4

Mean (±S.E.) of muscle force, MU force and number, muscle fibre size and number in cross-reinnervated TA muscles after immediate nerve cross suture (no delay) are compared to normal TA muscles in unoperated rats (control). Cross-reinnervated muscles developed less tetanic forces than control muscles ($p < 0.05$) and contained fewer muscle fibres ($p < 0.05$) (Fig. 3.6F). There were no other significant differences between the control and cross-reinnervated muscles.

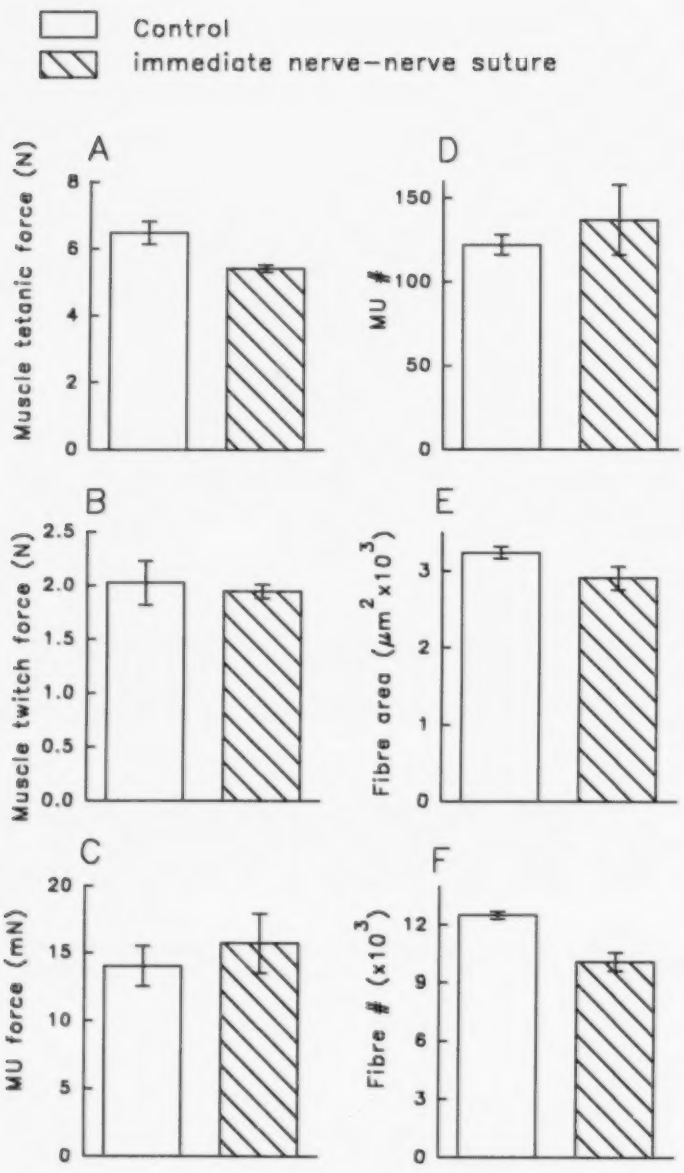

Control
immediate nerve–nerve suture
A
Muscle tetanic force (N)
0
2
4
6
8
D
MU #
0
50
100
150
B
Muscle twitch force (N)
0.0
0.5
1.0
1.5
2.0
2.5
E
Fibre area (μm² x10³)
0
1
2
3
C
MU force (mN)
0
5
10
15
20
F
Fibre # (x10³)
0
3
6
9
12

Fig. 3.5

Low and high power photomicrographs of cross-sections of a control (A, B), cross-reinnervated TA muscle after immediate nerve suture (C,D), and cross-reinnervated TA muscle after N-N suture following long-term axotomy (12 months) (E,F) stained for mATPase with acid pre-incubation. The total cross sectional area of reinnervated muscle after prolonged axotomy was similar to those after both immediate nerve suture and control. Reinnervated muscle fibres of the same histochemical types tended to clump but their size was not visually different from the control. The fibres in ascending intensity were type IIA (lightest), IIB (faint), Fint (intermediate), and type I (dark).

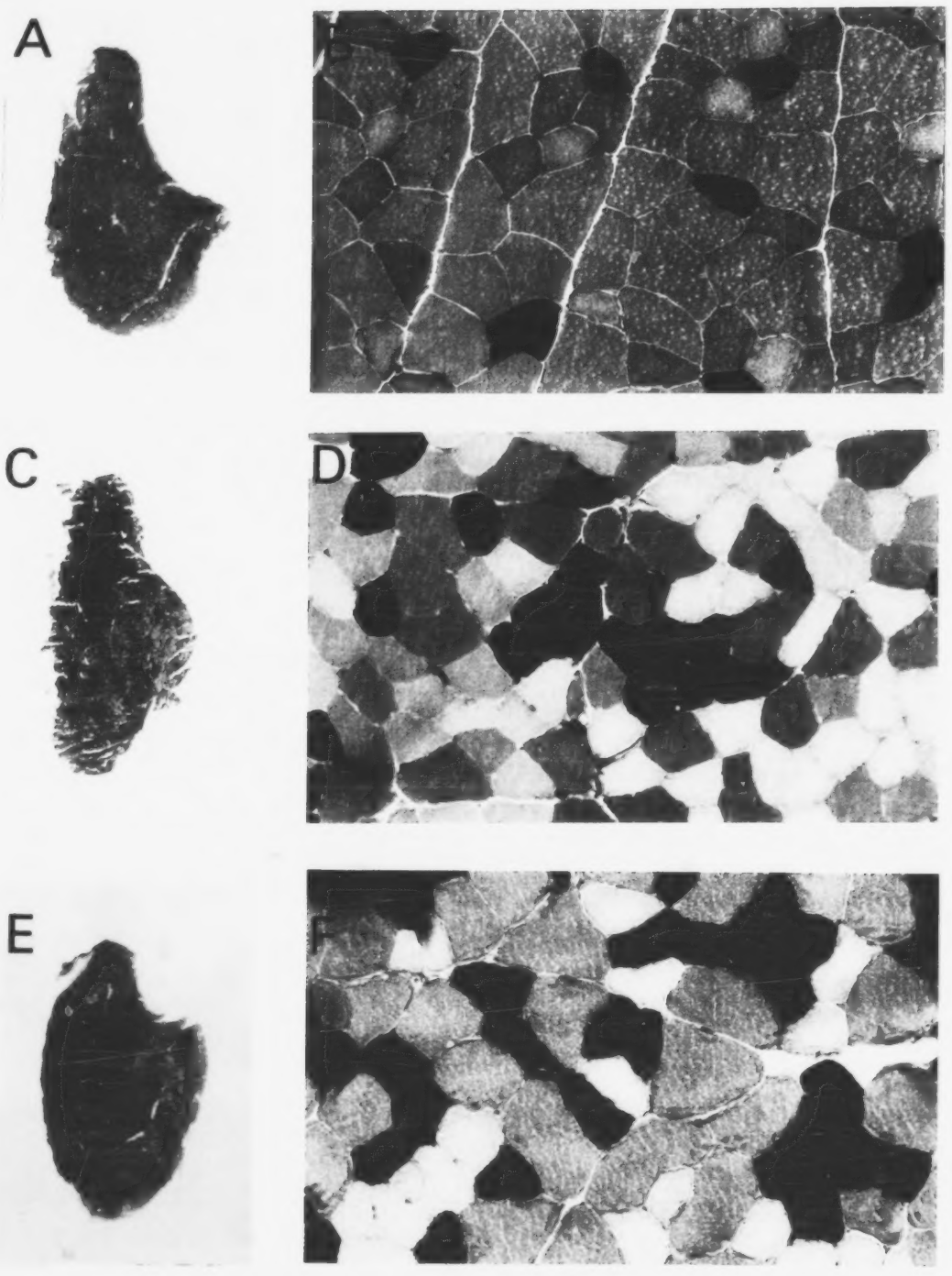

A
D
C
E
F
2 mm
50 μm

Fig. 3.6

Frequency histograms of CSA of muscle fibres in one normal TA muscle (A) and one cross-reinnervated TA muscle after immediate nerve suture (B) and one cross-reinnervated TA muscle after 12 month TIB axotomy (C). The mean muscle fibre CSA ($\pm$S.E.) (vertical line) was 2401 $\pm$47 μm^2 (A); 2853 $\pm$49 μm^2 (B), and 2616 $\pm$59 μm^2 (C).

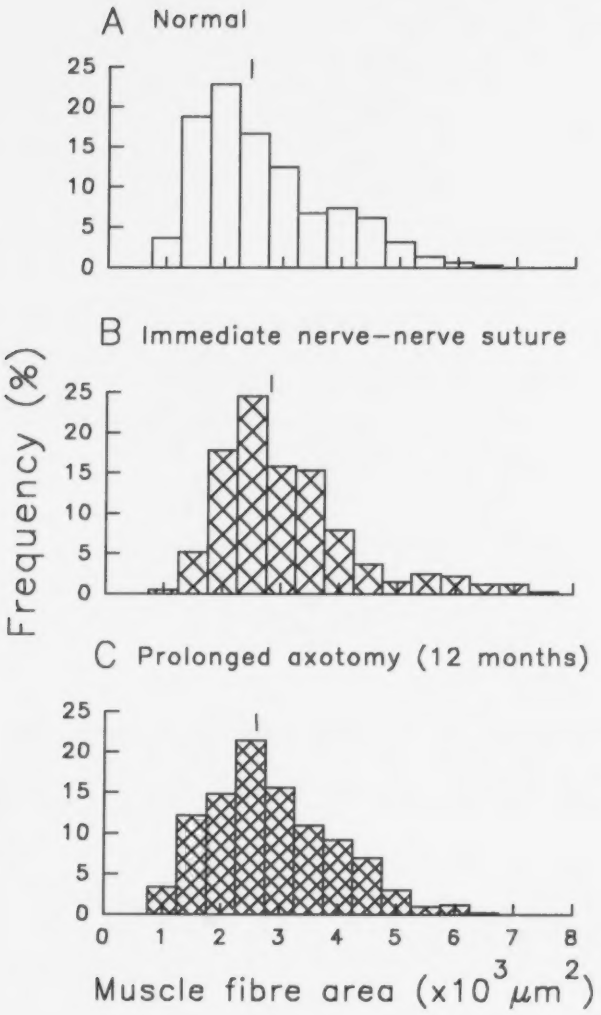

A Normal
B Immediate nerve–nerve suture
C Prolonged axotomy (12 months)
Frequency (%)
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8
Muscle fibre area (x10^3 μm^2)

Fig. 3.7

Mean (±S.E.) of muscle force (A), MU number (B), MU force (C), innervation ratio (D), total number of muscle fibre (E), and size of muscle fibres (F) in cross-reinnervated TA muscle plotted as a function of the duration of TIB axotomy prior to TIB-CP cross suture. Regression lines fitted to the plots in A, E, F, were not significantly different form zero showing that muscle force, muscle fibre number and muscle fibre area are not affected by prolonged axotomy. In contrast, the number of the reinnervated MUs declined (B) with a parallel increase in MU force (C) and innervation ratio (D). The decline and increases were well fitted by exponential curves with time constants of 40, 45, and 57 days.

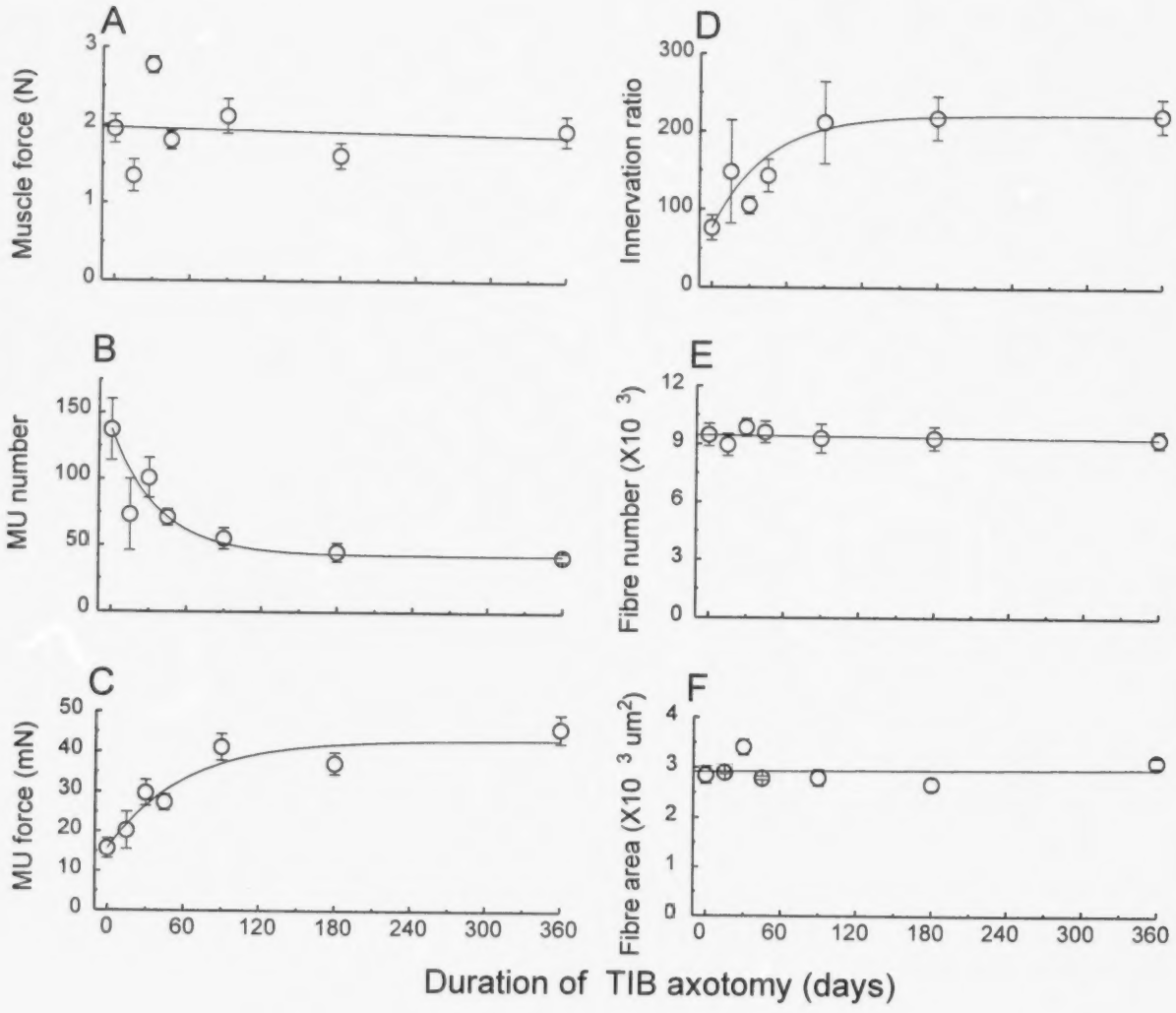

A
Muscle force (N)
3
2
1
0
D
Innervation ratio
300
200
100
0
B
MU number
150
100
50
0
E
Fibre number (X10 3)
12
9
6
3
0
C
MU force (mN)
50
40
30
20
10
0
F
Fibre area (X10 3 um2)
4
3
2
1
0
0
60
120
180
240
300
360
Duration of TIB axotomy (days)

Fig. 3.8

Means (± S.E.) of several muscle and MU parameters in cross-reinnervated TA muscles after long-term axotomy (> 6 months) and after immediate nerve cross suture. After prolonged axotomy, the total number of MUs decreased to 35% of the control (A); MU twitch force increased (B) because more muscle fibres are innervated by each axon (C). As a result of enhanced branching, as many muscle fibres were reinnervated as after immediate nerve repair (D) and the reduction in MU number is well compensated. In both cases, muscle fibres had completely recovered their sizes (E) and muscles had completely recovered their forces (F).

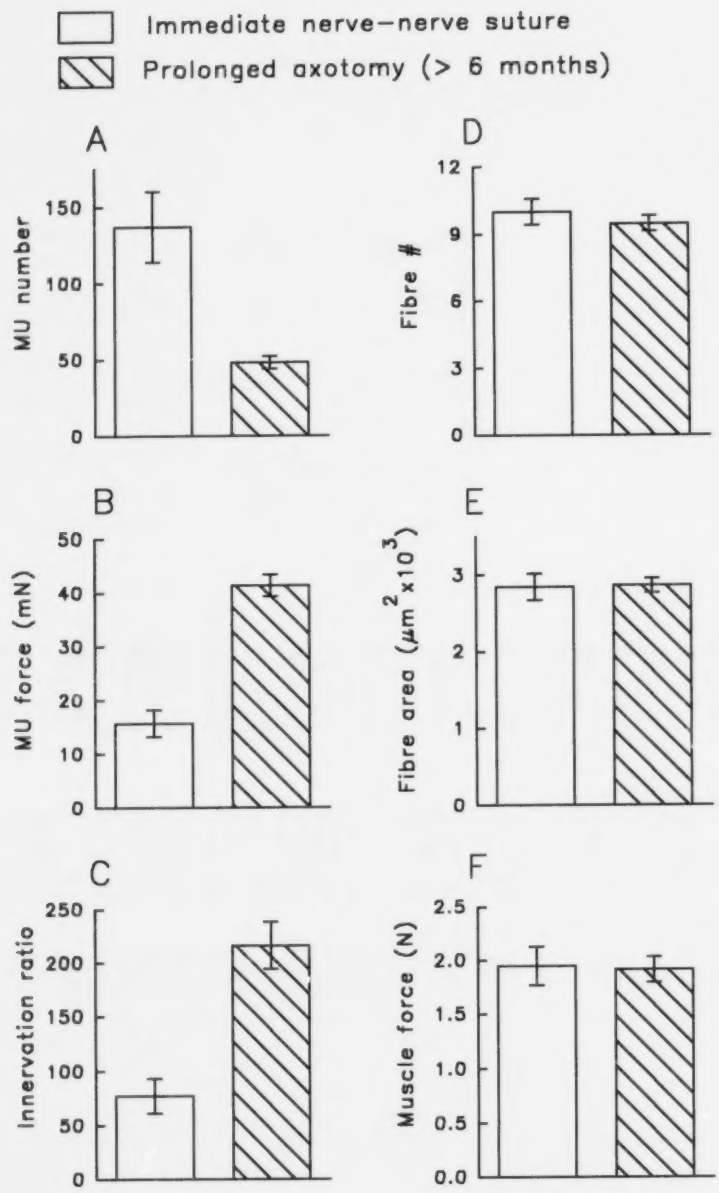

Immediate nerve–nerve suture
Prolonged axotomy (> 6 months)
A
MU number
150
100
50
0
D
Fibre #
12
9
6
3
0
B
MU force (mN)
50
40
30
20
10
0
E
Fibre area ($\mu m^2 \times 10^3$)
3
2
1
0
C
Innervation ratio
250
200
150
100
50
0
F
Muscle force (N)
2.5
2.0
1.5
1.0
0.5
0.0

Fig. 3.9

Mean($\pm$S.E.) time to peak twitch contractions (A) and proportion of slow muscle fibres (B) in cross-reinnervated TA muscles as a function of the duration of TIB axotomy prior to TIB-CP cross-suture. Muscle contraction speed did not change as a result of progressively longer duration of axotomy despite a small but significant increase in the proportion of slow muscle fibres ($p < 0.05$). The slopes ($\pm$ S.E.) of the regression lines in A and B were 0.004 ± 0.004 and 0.0092 ± 0.003.

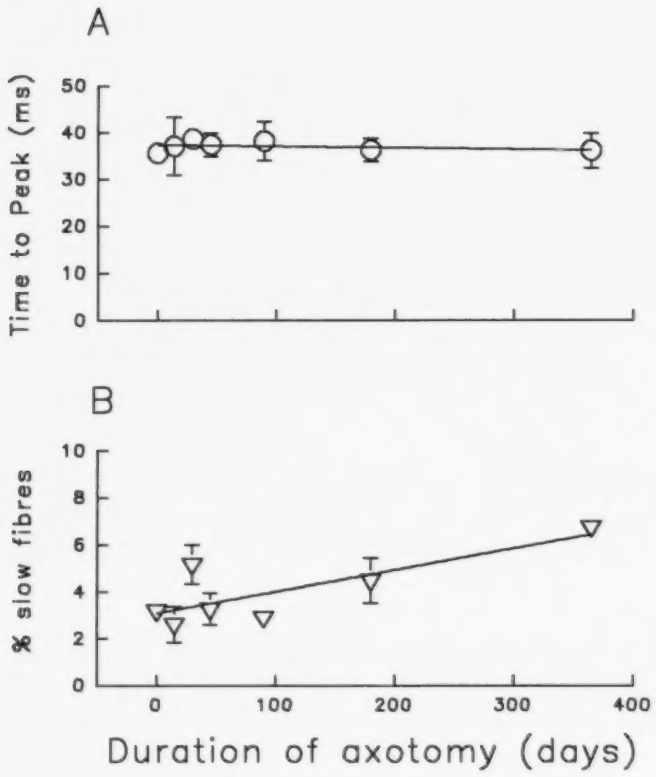
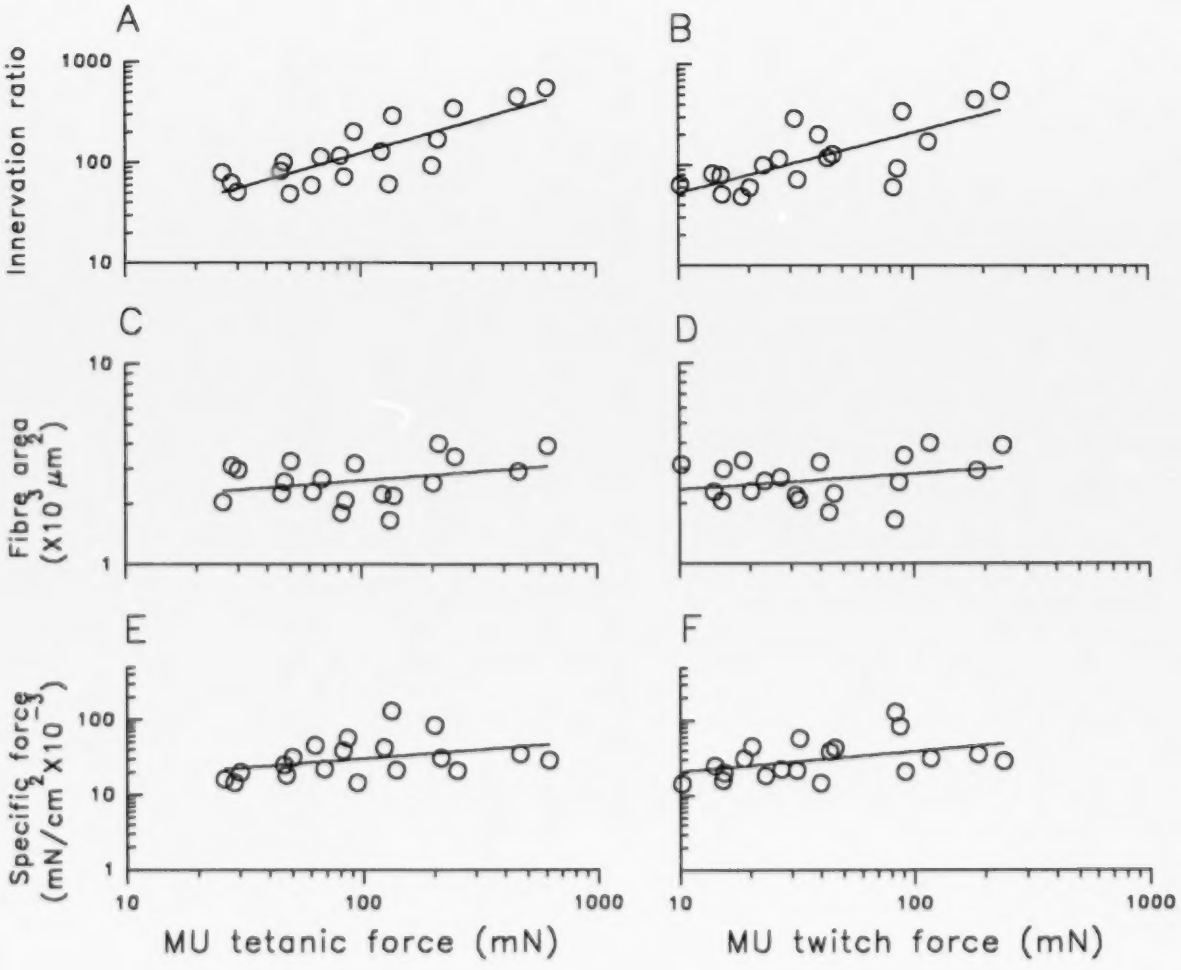

A
Time to Peak (ms)
50
40
30
20
10
0
B
% slow fibres
10
8
6
4
2
0
0
100
200
300
400
Duration of axotomy (days)

Fig. 3.10

Relationship of the number (innervation ratio, IR), mean cross sectional area (CSA), and specific force (SF) of the muscle fibres in single MUs with MU tetanic force (A,C,E) and twitch (B,D,F) forces on double logarithmic scales. Regression lines had slopes of 0.60±0.13 for IR and tetanic force and 0.67 ± 0.12 for IR and twitch force. Two slopes were not different from each other but were significantly different from zero. This shows that MU twitch force is as good as tetanic force in representing MU IR and that the IR is a major contributor to MU force. MU tetanic and twitch forces were not correlated with mean CSA [with slopes (± S.E) of 0.07 ± 0.08 and 0.1 ± 0.08] and SF [with slopes (± S.E.) of 0.36 ± 0.17 and 0.34 ± 0.17] showing that CSA and SF do not contribute significantly to the range in MU force.


A
B
C
D
E
F
Innervation ratio
1000
100
10
Fibre area (X10^{3} μm^{2})
10
1
Specific force (mN/cm^{2}X10^{-3})
100
10
1
10
100
1000
MU tetanic force (mN)
MU twitch force (mN)

CHAPTER 4

Effects of Long-term Denervation of the Rat Tibialis Anterior Muscle on Tibial Nerve Regeneration

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...2...Muscle and MU force Recordings
...3...Single MU isolation and glycogen depletion
...4...Histochemistry
...5...Data analysis
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.b...Muscle fibre number and size
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4.I...INTRODUCTION

Primary suture of proximal nerve lesions and delayed nerve repair are generally associated with very poor functional outcome (Merle, Amend, Cour, Foucher & Michon, 1986). For example, patients may not recover any useful hand function after secondary suture of high median and ulnar nerve injuries despite microsurgical repair (Birch & Raji, 1991). However the basis for the poor functional recovery after delayed surgical repair or injuries which require nerves to regenerate over long distance before reinnervating denervated targets is not well understood.

The poor recovery of motor function has generally been attributed to deterioration of denervated muscles, inability to accept innervation from regenerating motor axons, and inability to recover from denervation atrophy (Gutmann & Young, 1944; Gutmann, 1948; reviewed by Sunderland, 1978; Irintchev, Draguhn & Wernig, 1990; Terzis & Smith, 1990). However, delayed nerve repair may compromise the success of nerve regeneration and muscle reinnervation for several reasons. These include a reduction in the capacities of 1) injured axons to regenerate after axotomy, 2) distal nerve stumps to provide trophic and substrate support to the elongating axons and 3) denervated muscle to accept reinnervation and recover from denervation atrophy.

The effects of prolonged axotomy and denervation can be studied independently by a surgical nerve cross-anastomosis paradigm in which either a nerve is cut (axotomized) and later promoted to reinnervate a freshly denervated muscle via its nerve sheath

(prolonged axotomy) or a nerve is cut and later a foreign nerve is sutured to cross-reinnervate the long-term denervated muscle (prolonged denervation) (Holmes & Young, 1942; Gutmann & Young, 1944; Chapter 2). Using this paradigm and combined physiological and histochemical methods to quantify the number of motor axons which reinnervate muscles (*i.e.* motor unit number) after delay and the number of muscle fibres each motor axon has reinnervated (*i.e.* innervation ratio and MU size), we showed that the regenerative capacity of injured nerves is significantly reduced by prolonged axotomy (Chapter 3). In the present study, we used the same paradigm to quantify, for the first time, the effects of prolonged muscle denervation on nerve regeneration and muscle reinnervation. These studies extend the elegant morphological studies of Young and colleagues (Holmes & Young, 1942; Gutmann & Young, 1944) in demonstrating that deterioration of intramuscular nerve sheaths is the major factor limiting muscle reinnervation and recovery and confirm previous findings that reinnervated muscle fibres fail to recover fully from denervation atrophy after long-term denervation (Gutmann, 1948; Irinchev *et al.*, 1990). These findings have been presented in abstract form (Fu & Gordon, 1993a; Fu & Gordon, 1994).

4.II...METHODS

4.II.1...Surgical procedures

Cross-suture of tibial (TIB) nerve and common peroneal (CP) nerve was performed in 28 adult, female Sprague-Dawley rats under sodium pentobarbital anaesthesia and aseptic conditions. As shown in Fig. 4.1, the right tibialis anterior (TA) muscle was denervated by cutting the CP nerve and ligating the nerve to prevent regeneration. At the same time, all the other muscles innervated by CP nerve except TA and extensor digitorum longus (EDL) were removed from the anterior muscle compartment of hindlimb. Care was taken to avoid damaging the blood supply of the TA muscle. Immediately or up to 1 year after TA muscle denervation, the right TIB nerve was freshly cut and its proximal stump was sutured to the CP distal stump to cross-reinnervate the freshly or long-term denervated TA muscle. The proximal stump of the CP nerve was electrically stimulated to ensure that there was no CP nerve regeneration.

At least 6 months after the TIB-CP cross suture, the success of nerve regeneration and muscle reinnervation was evaluated in the final experiment as described in detail previously (Chapter 2). Rats were again anaesthetized under sodium pentobarbital and their blood volume was maintained by hourly *i.v.* injection of 5% dextrose-saline solution via a vein cannula. The trachea was cannulated for mechanical ventilation when necessary. The TA muscle was isolated by denervating all other hip, tail and hindlimb muscles. Stimulating electrodes were inserted into the muscle beneath the sciatic nerve.

Surface EMG electrodes were sewn on to the TA muscle fascia. Spinal ventral roots (L4-L6) were exposed by laminectomy. The isolated TA muscle was attached to a custom-made force transducer for muscle and MU force recordings.

4.II.2...Muscle and MU force Recordings

Muscle tetanic and twitch forces were recorded under optimal muscle length in response to supra-threshold sciatic nerve stimulation. Ventral roots were teased into small filaments which contained only 3-10 motor axons innervating the TA muscle. Ventral root filaments were stimulated by gradually increasing voltage to progressively recruit single MUs as judged by all-or-none increments in twitch force and associated unique EMG signals (Jansen & Fladby, 1990; MaComas, 1991; Stein & Yang, 1991). Twitch forces of single MUs were obtained by digital subtraction. To obtain a representative sample, MU twitch forces in at least 30-40% of the total MU population were measured.

The mean MU twitch force was calculated and used to obtain the total number of MUs in the muscle by dividing the total muscle twitch force by the mean MU twitch force (see Data Analysis below).

4.II.3...Single MU isolation and glycogen depletion

Upon completion of MU force recording, one MU in each muscle was isolated for further characterization into fast fatigable (FF), fast fatigue resistant (FR), fast fatigue intermediate (FI), and slow on the basis of its contractile speed, "sag" and fatigability

(Totosy de Zepetnek, Zung, Erdebil & Gordon, 1992a). The criteria of a single unit isolation were an all-or-none twitch contraction and associated EMG response. A motor unit was selected for study only if the threshold voltage of its axons was lower than 10 volts and at least 10 times less than the thresholds of any other unit in the same filament. These criteria were rigidly met to keep a single MU for the 1-3 hours of recordings required for adequate depletion of the glycogen. The isolated single MU was repetitively stimulated (starting with 1 Hz) with trains of tetanic stimuli consisting of five pulses at 50 to 100 Hz. These methods have been described in detail in Totosy de Zepetnek *et al.*, (1992a).

4.II.4...Histochemistry

After the above electrophysiological recordings, the TA muscle was quickly removed, cut into three blocks, and frozen in isopentane cooled in liquid nitrogen. Muscle cross sections (10 μm) were cut and stained for Periodic Acid Schiff staining (PAS) and myosin ATPase, using both acid and alkaline pre-incubation (Totosy de Zepetnek, Zung, Erdebil & Gordon 1992b).

4.II.5...Data analysis

MU number and size. At least 30% of MUs in any single reinnervated muscle were sampled to obtain a representative mean MU twitch force. The number of MUs in

each muscle was estimated by dividing the whole muscle twitch force by the mean MU twitch force.

Muscle fibre number and size. The total number of muscle fibres in each muscle was estimated by multiplying the total muscle cross-sectional area (CSA) by muscle fibre density. Muscle CSA was measured on those cross-sections which contained the maximum number of muscle fibres. The muscle cross sections were carefully checked during the measurement to exclude as much fibrotic and fatty tissue as possible. Muscle fibres were counted in 0.63 mm^2 areas on muscle cross-sections in 6-9 regions which were located consistently in different muscles. Fibres within the 6-9 regions represented at least 10% of all the muscle fibres in each muscle. The fibre density was measured in all the 6-9 regions in order to take into account for regional differences in fibre size (Pullen, 1977; Parry & Wilkinson, 1991; Totosy de Zepetnek *et al.*, 1992*a*).

Two methods were used to obtain the mean muscle fibre CSA: 1) dividing the total muscle CSA by the total number of muscle fibres in each muscle and 2) directly measuring the fibre CSA of 500-1000 fibres within the 6-9 regions. Good agreement was found between two methods: with mean muscle fibre CSAs being 2912 ± 63 μm^2 and 2985 ± 166 μm^2 for indirect and direct measurement respectively in the same 4 rats. The indirect method was therefore used to acquire the mean muscle fibre CSA in all the control and cross-reinnervated TA muscles.

4.II.6...Statistics

Arithmetic means were calculated and shown with standard errors (mean±S.E). One-way analysis of variance (ANOVA) was applied to examine differences in muscle force, MU number, MU force, muscle and muscle fibre CSA and muscle fibre number between the control, cross-reinnervated muscles after immediate nerve repair and cross-reinnervated muscles after delayed nerve repair. Bonfferoni tests were used to detect any differences between all the possible combinations of paired conditions. The Kruskal-Willis test of rank order was used to examine the differences in the distribution of muscle fibre CSA between control and cross-reinnervated muscles. Regression lines were fitted using least square analysis. For all the above statistical analyses, p values of less than 0.05 were regarded as significant.

4.III...RESULTS

4.III.1...Muscle reinnervation after immediate and delayed nerve-nerve sutures

The micrographs in Fig. 4.2 show that cross-reinnervated muscles contained fewer and smaller muscle fibres, particularly when muscle denervation was prolonged for more than 6 months prior to nerve repair. As shown in the muscle fibre size distributions in Fig. 4.3, muscle fibres in a cross-reinnervated TA muscle fully recovered from denervation atrophy when TIB-CP cross-suture (N-N) was carried out within 45 days of denervation. However, reinnervated muscles fibres were significantly smaller when

denervation was prolonged beyond 6 months prior to N-N suture. As summarized in Fig. 4.4, a progressive decline in both the number (B) and size (C) of muscle fibres accounts for a smaller total muscle CSA (A) and muscle twitch force (D). However there was little change in mean MU twitch force (E). The most dramatic effect of prolonged denervation was a large decrease in the number of reinnervated MUs (F) and hence the number of regenerating motor axons which made functional contact with long-term denervated muscle.

A small trend for MU force to increase with prolonged muscle denervation (Fig. 4.4E) suggested that the few axons which made nerve-muscle contacts may have formed enlarged MUs to compensate for reduced MU numbers. However, because reinnervated muscle fibres were smaller than normal, MU force underestimated the actual number of muscle fibres per motoneurone.

Innervation ratio (IR), calculated by dividing total muscle fibre number by MU number, increased significantly to partially compensate for the reduction in MU number in the cross-reinnervated muscles (Fig. 4.5). The average increase of 3 fold is as great as for intact axons which sprout in partially denervated TA muscles (Gordon *et al.*, 1993) or regenerating axons which form enlarged MUs after immediate nerve repair (Chapter 2). Thus, prolonged denervation does not affect the ability of motor axons to form enlarged MUs. However, the upper limit of MU enlargement is insufficient to compensate for the 90% reduction in MU number. As a result, less than 50% of the muscle fibres were reinnervated when denervation was prolonged beyond 6 months (Fig. 4.4B).

The tetanic force developed by cross-reinnervated muscles after immediate N-N suture was similar to the contralateral control (5.4 ± 0.1 mN *vs.* 6.0 ± 0.2 mN). In contrast, the reduction in tetanic force after prolonged denervation was associated with hypertrophy of the contralateral TA muscle (Fig. 4.6) which presumably resulted from over-activity compensating for the "foot drop" in the experimental limb.

4.III.2...Muscle reinnervation after immediate and delayed nerve-muscle sutures

One possible explanation for the dramatic decline in the number of reinnervated MUs after prolonged denervation is that regenerating axons fail to elongate in deteriorating intramuscular nerve sheaths. Morphological studies show that regenerating axons may escape from the sheaths and grow directly on denervated muscle surface (Gutmann and Young, 1944). If this is the case, poor reinnervation of long-term denervated muscle after N-N suture should be mimicked by forcing nerves to regenerate outside the intramuscular nerve sheaths. This was achieved by suturing the TIB nerve directly to denervated TA muscle at least 8 mm away from the nerve entry point (N-M suture). N-M suture was carried out immediately after TA muscle denervation or after the same periods of prolonged denervation as in the experiments of N-N suture.

The success of reinnervation of short-term (<45 days) denervated muscles observed after N-N suture was not seen after N-M suture (Fig. 4.7 & 4.8). Even immediate N-M suture led to a significant reduction in the number of reinnervated MUs

(Fig. 4.8F), the associated decrease in muscle CSA (Fig. 4.8C), force (Fig. 4.8D) and muscle fibre number (Fig. 4.8A) (see also Chapter 2).

By forcing axons to regenerate outside the intramuscular nerve sheaths (N-M suture) we simulated poor reinnervation of long-term denervated muscle. This provided evidence to support the idea that poor reinnervation of long-term denervated muscles after N-N suture can be explained by escape of regenerating nerves from deteriorating intramuscular nerve sheaths and elongation on the denervated muscle surface.

To determine whether the growth substrate of denervated muscle also deteriorates with time, denervation was prolonged prior to N-M suture. The effects were dramatic. A two week delay prior to N-M suture reduced the number of reinnervated MUs to an average of 10 in comparison with 84 ± 4 for the same delay prior to N-N suture (Fig. 4.8F). Thus the growth substrate of denervated muscle deteriorates rapidly as compared with the denervated intramuscular sheaths. As a result of deterioration of the sheaths, reinnervated MU number was similarly low when N-N or N-M sutures were performed 6 months after TA muscle denervation.

We have compared the effects of N-N and N-M suture, and of immediate and prolonged nerve repair, in Fig. 4.9. After immediate nerve repair, regeneration (MU number, Fig. 4.9A) was significantly less successful after N-M suture than after N-N suture. The capacity of the few regenerated axons to form enlarged MUs after N-M suture (Fig. 4.9B) partially compensated for the reduction in MU number. As a result, almost as many muscle fibres were reinnervated (Fig. 4.9C) and reinnervated muscle force

recovered almost as well after N-N suture (Fig. 4.9E). Denervated muscle fibres recovered completely from denervation atrophy after both N-N and N-M sutures (Fig. 4.9D).

After delayed nerve repair and prolonged muscle denervation, MU number was equally reduced after N-N and N-M sutures to less than 10% of the number after immediate N-N suture (Fig. 4.9A). After delayed N-N suture, the capacity of motor axons to form enlarged MUs partially compensated for reduced MU number (Fig. 4.9B). However, the relatively poor reinnervation of denervated muscle fibres (4901 ± 830 and 10006 ± 470 after delayed and immediate N-N sutures respectively) combined with the failure of long-term denervated muscle fibres to fully recover from denervation atrophy (Fig. 4.9D) resulted in less than 25% muscle force compared to immediate N-N suture (Fig. 4.9E). After delayed N-M suture, IR did not increase to compensate for low MU number (Fig. 4.9B) with the result that reinnervated muscle force was less than that after delayed N-N suture. Thus long-term denervation reduces both the number of motor axons which successfully reinnervated the muscle and the capacity of motor axons to form enlarged MUs.

4.III.3...Relationship between IR and MU force after N-N and N-M sutures

Similar to previous findings in normal and self-reinnervated TA muscles (Totosy de Zepetnek *et al.*, 1992a), direct counts of MU fibres (IR) strongly correlated with MU tetanic force in cross-reinnervated muscles (Fig. 4.10A-C). In addition, the same

correlations were shown between IR and twitch force (Fig. 4.10D-F). Interestingly the slopes of the regression lines fitted to the data from reinnervated muscles after N-N and N-M sutures were the same, irrespective of short or long period of denervation. Thus, the relationship between IR and MU force did not change as a result of the reduction in the number of MUs after N-M suture or by prolonged denervation. However, after prolonged denervation, tetanic and twitch forces underestimate IR because muscle fibre CSA is lower than normal. This is seen as a shift of the regression lines to higher values of IR (Fig. 4.10C & F).

4.IV...DISCUSSION

The present study demonstrates that poor recovery of reinnervated muscles after prolonged denervation is due to 1) failure of most regenerating axons to reinnervate muscle and 2) inability of the few motor axons that do make connections to reverse denervation atrophy. The axons which were successful in making nerve-muscle connections reinnervate many more denervated muscle fibres than normal. However, axons failed to reinnervate all available muscle fibres because MU size cannot increase beyond a limit of 3-5 times normal.

4.IV.1...Good muscle recovery after short-term denervation

It has been suggested that delayed nerve repair may be beneficial for recovery of motor function (Brunetti, Carretta, Magni & Pazzaglia, 1985). Although we observed a trend for cross-reinnervated TA muscles to develop more force when they remained denervated for up to one month before nerve repair (see also Finkelstein, Dooley & Luff, 1992), the number of motor axons which made functional connections was not any higher than after immediate nerve repair (Fig. 4.3). Muscle fibres fully recovered from denervation atrophy in either case (Fig. 4.6). These findings suggest that endpoint measurements of muscle force may be misleading in judging the optimal time interval between nerve lesion and repair (neurorrhaphy) for the best outcome in terms of how many regenerating axons make synaptic contacts with denervated muscles. More extensive analysis of MU number and muscle fibre size indicates that benefits of a brief delay in nerve repair are minimal and that if any benefit may ensue, this involves a small increase in the number of muscle fibres supplied by each regenerating axons.

4.IV.2...Poor muscle recovery after prolonged denervation

More striking are the results that prolonged denervation was detrimental to both axonal regeneration and the recovery of reinnervated muscle fibres from denervation atrophy. Muscles developed less than 10% of normal forces, primarily because progressively fewer muscle fibres were reinnervated by regenerating axons when the duration of denervation was prolonged prior to N-N suture. Less than 50% of muscle

fibres were reinnervated due to the small number of MUs (10% of normal) and the limit of MU size to 3-5 times normal (Fig. 4.4). The poor recovery of muscle force, weight and girth (see also Finkelstein *et al.*, 1993) was further exacerbated by the incomplete reversal of denervation atrophy in the reinnervated muscle fibres which recovered only 50% of normal fibre size (Fig. 4.4).

4.IV.3...Reduction in reinnervated muscle fibres after prolonged denervation

The reduction in the number of reinnervated muscle fibres could result from 1) a reduction in the number of viable muscle fibres available for reinnervation (Anzil & Wernig, 1989), 2) a reduction in the ability of long-term denervated muscle to accept reinnervation (Gutmann & Young, 1944), and/or 3) a reduction in the number of axons which succeed in elongating to the denervated muscle fibres and making functional nerve-muscle connections.

Although long-term denervated muscle fibres undergo extensive degeneration and atrophy (Gutmann & Young, 1944; Anzil & Wernig, 1989; Schmalbruch, Al-Amood & Lewis, 1991), regeneration of new fibres from satellite cells has been reported to be sufficient to maintain the normal number of viable muscle fibres even 10 months after denervation (Mussinin, 1987; Schmalbruch *et al.*, 1991). Thus, unless the satellite pool is exhausted, a reduction in the number of viable muscle fibres is unlikely to be a major contributing factor to the reduced number of reinnervated muscle fibres after delayed nerve repair (Gutmann & Zelena, 1962; Anzil & Wernig, 1989). Our findings that the IR of the

few reinnervated MUs increased to the same 5-fold upper limit for MU enlargement as after immediate nerve repair (Rufuse *et al.*, 1994a; Chapter 2) and after partial denervation (Rafuse, Gordon & Orozco, 1992; Gordon, Yang, Stein & Tyreman, 1993) further argue against the idea that there are few viable muscle fibres to reinnervate. Since each motor axon supplies as many muscle fibres as possible, reduction in the number of regenerating axons which succeed in reinnervating denervated muscle fibres is a more likely explanation for the poor reinnervation. Even in the short-term denervated muscles where degenerative changes were minimal, we observed the same small number of reinnervated MUs after N-M suture which was associated with significant reduction in the number of regenerating nerve fibres. Thus poor reinnervation of long-term denervated muscle is far more likely due to failure of many regenerating axons to elongate and/or make synaptic contacts with denervated muscle fibres than a reduced viability of denervated muscle fibres.

The finding that enlarged MUs in reinnervated muscles included as many muscle fibres as possible also argues against the idea that long-term denervated muscles fail to accept reinnervation (see Gutmann & Young, 1944). Again it is more likely that all muscle fibres are not reinnervated because only a few motor axons made connections even though many more muscle fibres than normal were reinnervated by each axon because of compensatory MU enlargement. Our findings are in agreement with previous studies showing that after prolonged denervation the number of reinnervated muscle fibres is reduced when fewer nerve fibres regenerate (Anzil & Wernig, 1989) and remains the same

as normal when the number of successfully regenerated nerve fibres is maximized by repeated freezing of the sciatic nerve (Irintchev *et al.*, 1990).

In view of the above arguments, the most parsimonious explanation for poor reinnervation of long-term denervated muscles is a reduction in the number of regenerating axons which grow back to denervated muscle fibres to make nerve-muscle contacts.

4.IV.4...Reduction in the number of reinnervated MUs after prolonged denervation

Our finding that the poor regenerative outcome after delayed nerve repair by cross-union of nerve stumps is mimicked after direct muscle neurotization (after N-M suture) strongly suggests that poor nerve regeneration is likely due to progressive deterioration of intramuscular nerve sheaths with prolonged denervation. The normal route for nerve regeneration is the intramuscular nerve sheaths which guide regenerating motor axons to the denervated endplates (Gutmann & Young, 1944; Sanes, Lawrence, Marshall & McMahan, 1978; Kuffler, 1986). The regenerating axons grow in the interface between Schwann cells and their basal laminae both of which provide the growth substrates and trophic support for the regenerating axons (Nathaniel & Pease, 1963; Martini, 1994). There are a number of possible reasons why the intramuscular nerve sheaths may progressively fail to support and guide regenerating axons back to long-term denervated muscle. (1) The initial proliferation of Schwann cells is not maintained and the number of Schwann cells may decrease to a level at which adequate substrate and trophic support is no longer available (Weinberg & Spencer, 1978; Pellegrino & Spencer, 1985; Salonen,

Lehto, Kalimo, Penttinen & Aho, 1985; Salonen, Peltonen, Roytta & Virtanen, 1987; You, Raji & Gordon, 1994). (2) The basal lamina, which is not renewed in the absence of Schwann cell-axonal contact (Bunge, Williams & Wood, 1982), may fragment and fail to guide regenerating axons (Gianini & Dyck, 1990). (3) Collagenation of endoneurial tubes may obstruct regeneration of axons (Sunderland & Bradley, 1950a,b). All these factors could reduce the number of axons reaching the denervated muscle via the intramuscular nerve sheath. Evidence from morphological studies indicates that regenerating axons escape from deteriorating intramuscular nerve sheaths and grow directly on the surface of denervated muscle fibres (Gutmann & Young, 1944). The present experiment strongly support this finding in that the effect of long-term denervation in reducing MU number was mimicked by excluding regenerating axons from the intramuscular nerve sheaths. We found that far fewer axons made functional nerve-muscle contacts after N-M suture than after N-N suture (Fig. 4.8). These findings show that denervated muscle surface is a poor substrate for nerve growth as compared to the intramuscular nerve sheath (see also Chapter 2). Evidently cell adhesion molecules and ECM proteins expressed on the denervated muscle surface (Covault, Cunningham & Sanes, 1987) do not provide as favorable substrate or trophic support for axon elongation as Schwann cells and their basal laminae. Even in freshly denervated muscles, less than 50% of regenerating axons made nerve-muscle contracts after N-M suture as compared to N-N suture (Fig. 4.7). The finding that a delay of 2 weeks prior to N-M suture leads to further reduction in the number of reinnervated MUs shows that the growth substrate

provided by the denervated muscle rapidly deteriorates. The number of reinnervated MUs fell to less than 10% if muscle denervation was prolonged. Eventually the number of reinnervated MUs after delayed N-N suture fell to the same level as after N-M suture (Fig. 4.8) suggesting that deterioration of the intramuscular nerve sheaths progresses more slowly than that of the growth substrate provided by the denervated muscle.

Regenerating axons which are forced to grow outside the intramuscular nerve sheaths by direct neurotization (N-M suture) elongate along the denervated muscle fibres. However, they may still be strongly attracted by the intramuscular nerve sheaths (Chapter 2). Axonal regeneration appears to deteriorate rapidly with prolonged denervation suggesting that expression of high levels of N-CAM, for example, may not be sustained. The production of ECM molecules such as fibronectin and heparin-sulfate proteoglycan may fall with time, although this possibility does not seem to have been studied. An alternative is that the Schwann cells which have migrated from the endplate region also fail to support regenerating axons. In addition, the intramuscular nerve sheaths may fail to attract and support axonal regeneration as they deteriorate.

4.IV.5...Incomplete recovery of muscle fibre size after prolonged denervation

Failure of reinnervated muscle fibres to recover normal size suggests that muscle atrophy is only partially reversible after prolonged denervation, possibly as a result of exhaustion of satellite cells. After long periods of denervation, viable muscle fibres are very small (Gutmann, 1948; Sunderland & Ray, 1950; Schmalbruch *et al.*, 1991). Since

postnatal growth of muscle fibre diameter relies on satellite cells as a source of muscle fibre nuclei (Moss & LeBlond, 1971; Mazanet & Franzini-Armstrong, 1986), it is likely that reversal of denervation atrophy by reinnervation also depends on satellite cells for incorporation of new nuclei. Even if the number of satellite cells is sufficient to maintain viable muscle fibres for reinnervation, long-term denervation may severely deplete the satellite cell pool and thereby reduce the number of satellite cells available for recovery of reinnervated muscle fibre size. This possibility is being tested by irradiation of the denervated muscle after muscle reinnervation.

An additional factor which may limit muscle recovery from denervation atrophy is endomysial fibrosis and connective tissue accumulation. Both may physically limit the ultimate size of muscle fibres (Gutmann & Young, 1944; Savolainen, Myllyla, Myllyla, Vihko, Vaananen & Takala, 1988).

4.IV.6..Conclusions

Prolonged denervation profoundly reduces the number of successfully regenerated motor axons. In addition, muscle fibres progressively fail to recover from denervation atrophy with increasing duration of muscle denervation prior to nerve repair. The combined effects of the reduction in the number of regenerated axons and incomplete recovery of muscle fibres from denervation atrophy drastically compromise the force capacity of reinnervated muscle. Thus, prolonged denervation due to delayed nerve repair is very detrimental to functional recovery.

These findings have implications in surgical repair of injured nerves. Poor recovery of long-term denervated muscle provides the basis for many clinical observations that distal musculature is poorly, if at all, reinnervated after proximal nerve lesions and/or delayed nerve repair in both of which nerve regeneration will take many months (Sunderland, 1978; Terzis & Smith, 1990). Our results suggest that deterioration of the growth environment in the intramuscular nerve sheaths (Gutmann & Young, 1944) is likely the major cause for poor nerve regeneration and muscle reinnervation. Exhaustion of the satellite cell population may account for incomplete recovery of reinnervated muscle fibre size. Thus efforts should be devoted to optimizing the growth environment in the intramuscular nerve sheaths and denervated muscles, in addition to minimizing the effects of denervation atrophy. The present study also shows that the surface of the denervated muscle, particularly after prolonged denervation, is a poor substrate for nerve growth. Thus direct neurotization of muscle is not likely to be successful unless regenerating axons can be directed into the intramuscular nerve sheaths.

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Fig. 4.1

Diagrammatic illustration of surgical procedures. A: The sciatic nerve normally branches into the common peroneal (CP) nerve and posterior tibial (TIB) nerve. The CP nerve innervates muscles in the anterior compartment of the hindlimb including the tibialis anterior (TA) muscle (darkened). The TIB nerve innervates the intrinsic muscles of the foot (not shown). B: The CP nerve was cut and its proximal stump was ligated and sutured to the biceps femoris to prevent regeneration. C: Up to one year later, the TIB nerve was freshly cut and its proximal stump was sutured to the previously cut distal CP stump to reinnervate the freshly or prolonged denervated TA muscle. Before the TIB-CP cross suture the proximal CP stump was stimulated to ensure that there was no regeneration.

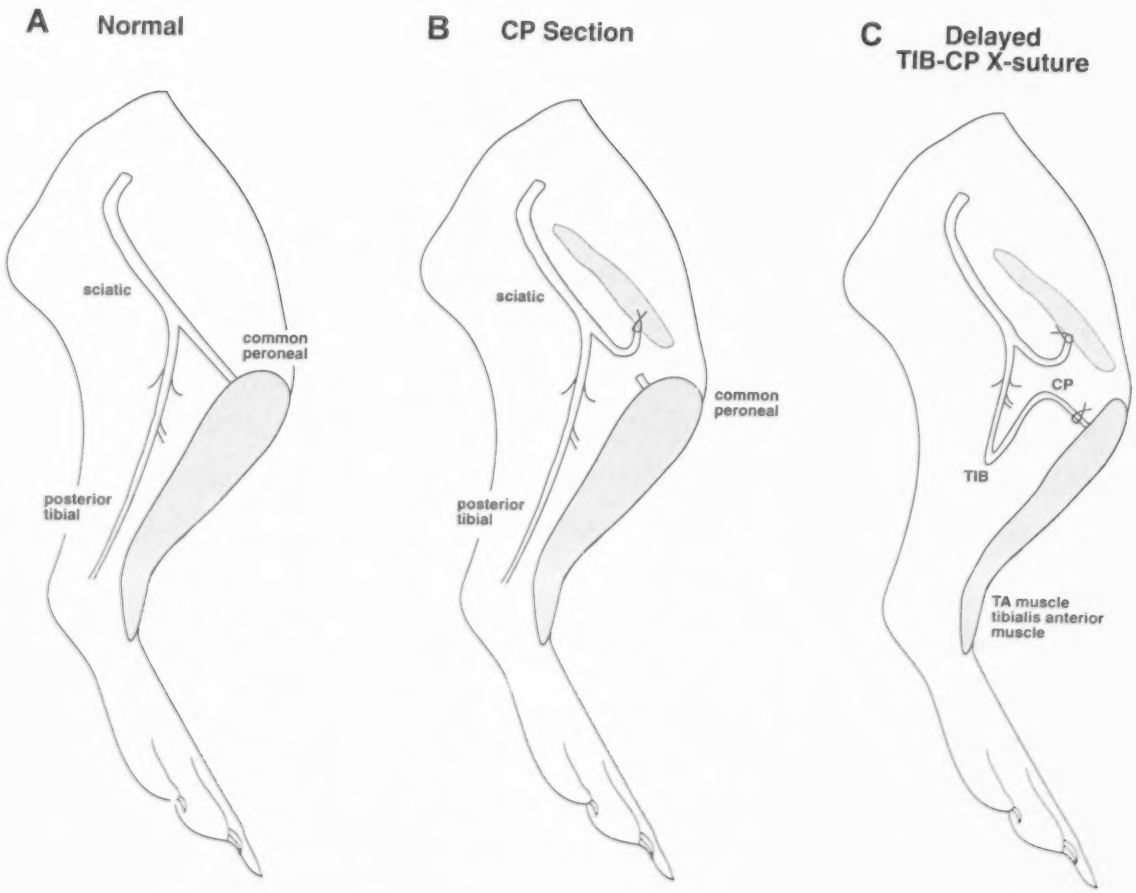

A Normal
sciatic
common peroneal
posterior tibial
B CP Section
sciatic
common peroneal
posterior tibial
C Delayed TIB-CP X-suture
CP
TIB
TA muscle tibialis anterior muscle

Fig. 4.2

Low and higher power photomicrographs of cross sections of control (A,B), cross-reinnervated muscle after short-term (1 month) denervation (C,D) and long-term (12 month) denervation (E,F) prior to nerve-nerve suture. The size of the reinnervated muscle and its muscle fibres was similar to the control after short-term denervation prior to nerve repair but was much smaller than the control when denervation was prolonged for 12 months prior to nerve repair.

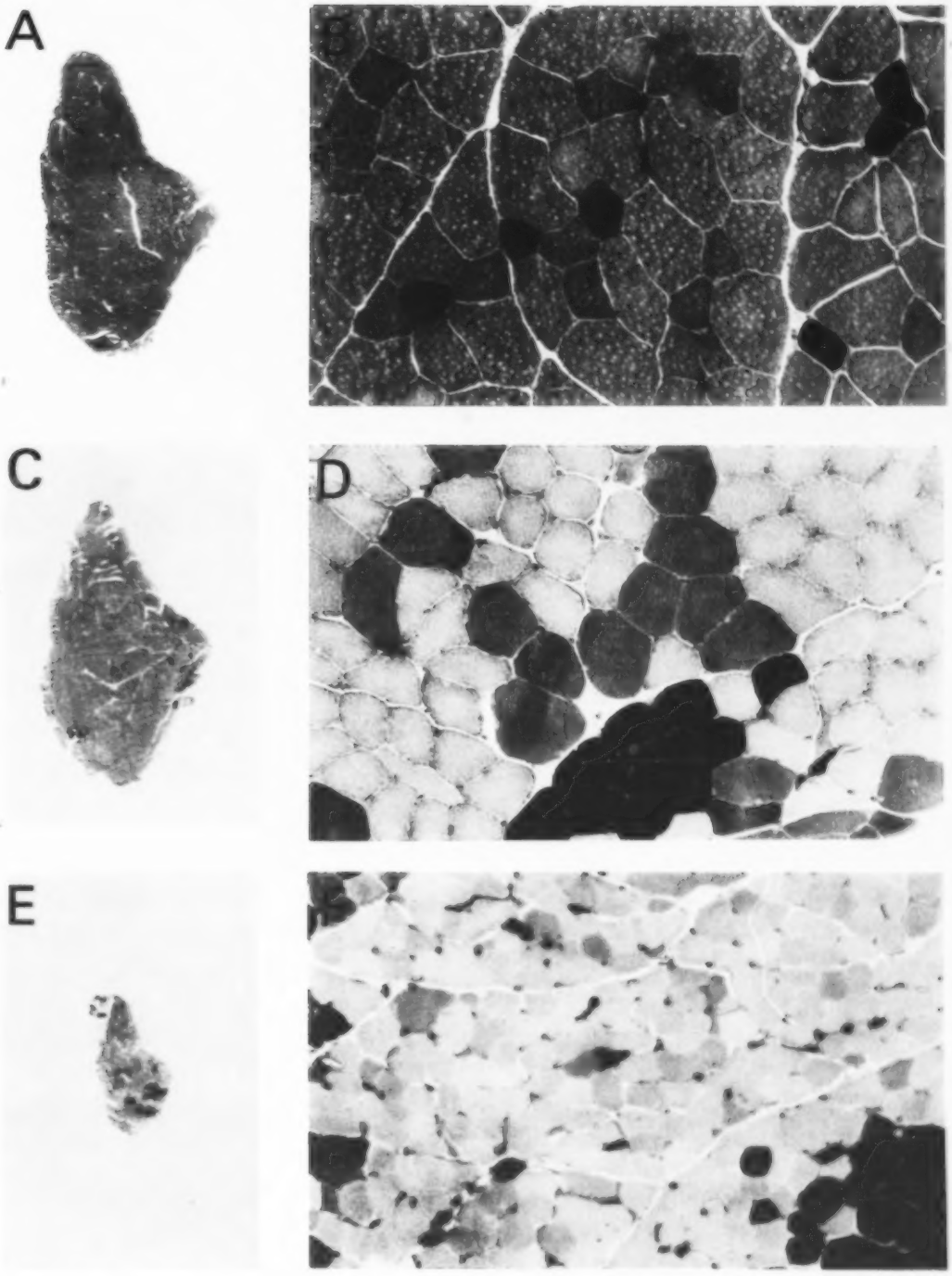

A
B
C
D
E
2 mm
50 μm

Fig. 4.3

Frequency distribution of muscle fibre cross-sectional area in one control muscle (A), one reinnervated muscle after short-term denervation (B), and one reinnervated muscle after long-term denervation (C) prior to nerve-nerve suture. Mean (± S.E.) muscle fibre area was 2401 ± 47 μm^2 (A), 2503 ± 40 μm^2 (B), and 1031 ± 37 μm^2 (C).

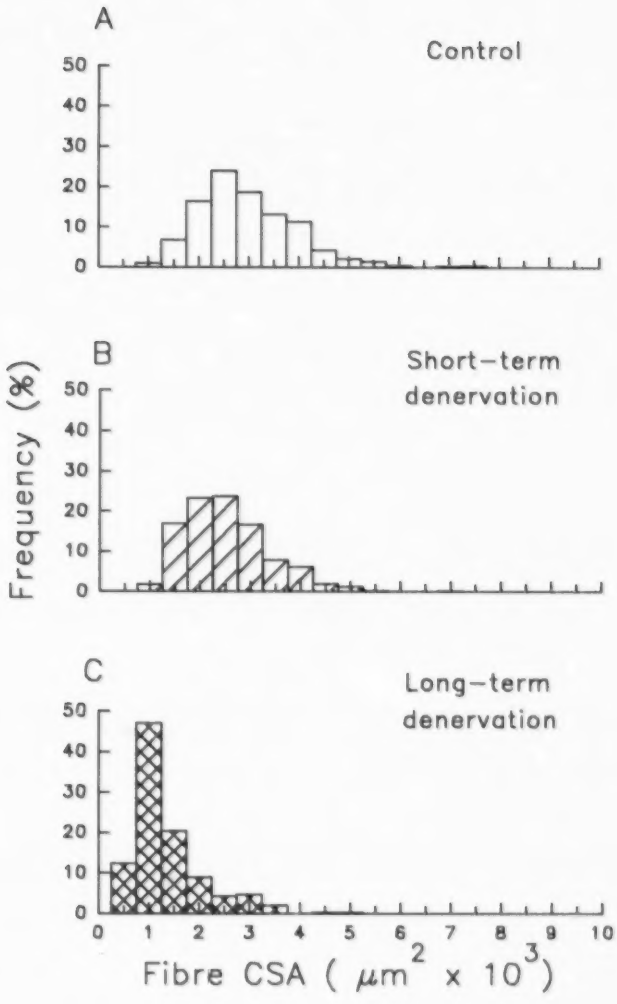

A
Control
B
Short–term
denervation
C
Long–term
denervation
Frequency (%)
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10
Fibre CSA (μm² x 10³)

Fig. 4.4

Mean (± S.E.) muscle cross-sectional area (CSA) (A), total muscle fibre number (B), muscle fibre CSA (C), twitch force (D), MU twitch force (E), and MU number (F) plotted as a function of the duration of muscle denervation prior to N-N suture.

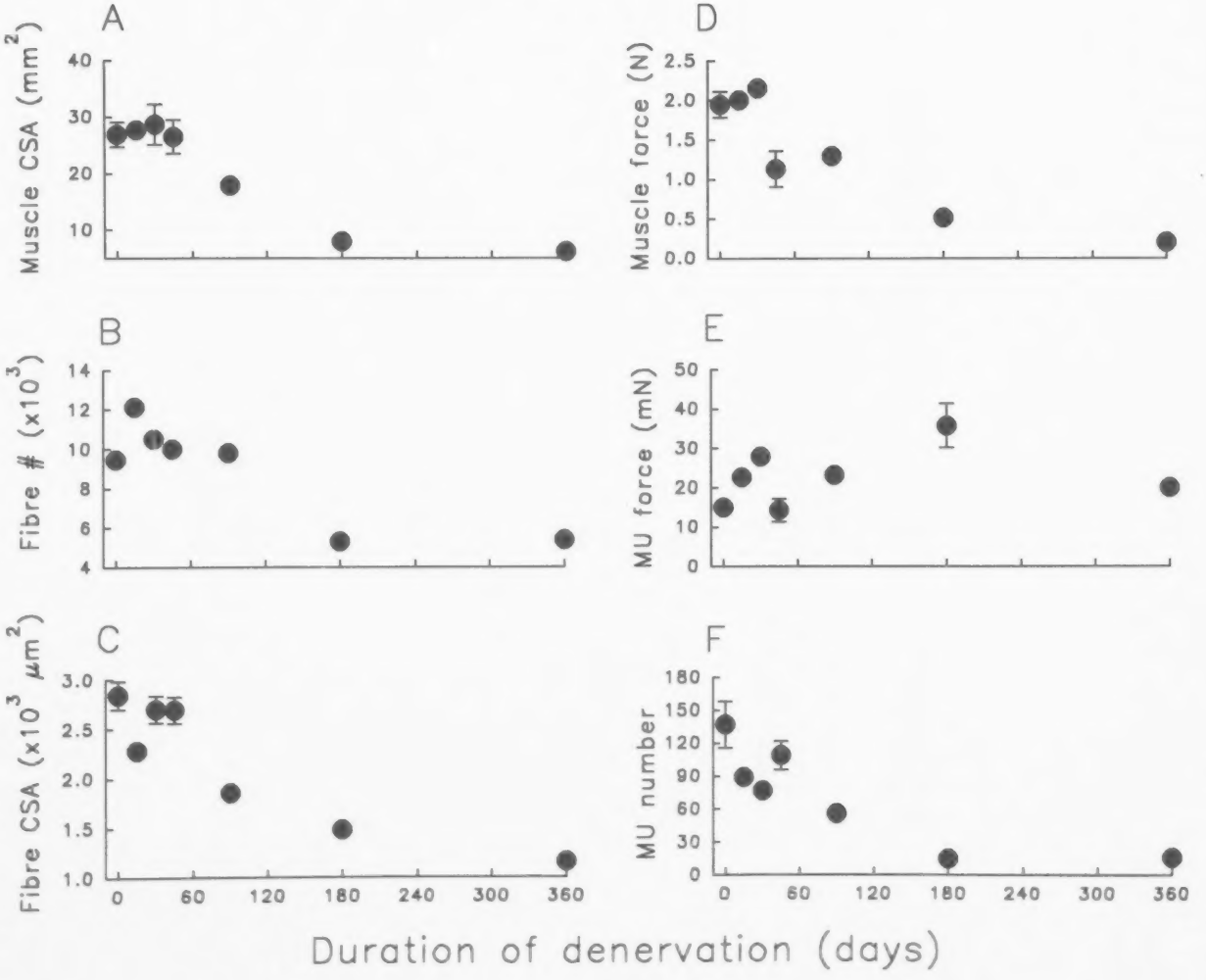

A
Muscle CSA (mm^2)
40
30
20
10
B
Fibre # (x10^3)
14
12
10
8
6
4
C
Fibre CSA (x10^3 μm^2)
3.0
2.5
2.0
1.5
1.0
D
Muscle force (N)
2.5
2.0
1.5
1.0
0.5
0.0
E
MU force (mN)
50
40
30
20
10
0
F
MU number
180
150
120
90
60
30
0
0
60
120
180
240
300
360
Duration of denervation (days)

Fig. 4.5

Mean (± S.E.) number of muscle fibres reinnervated by each motoneurone (IR) (the left Y-axis) and MU number (the right Y-axis) plotted as a function of the duration of muscle denervation prior to N-N suture.

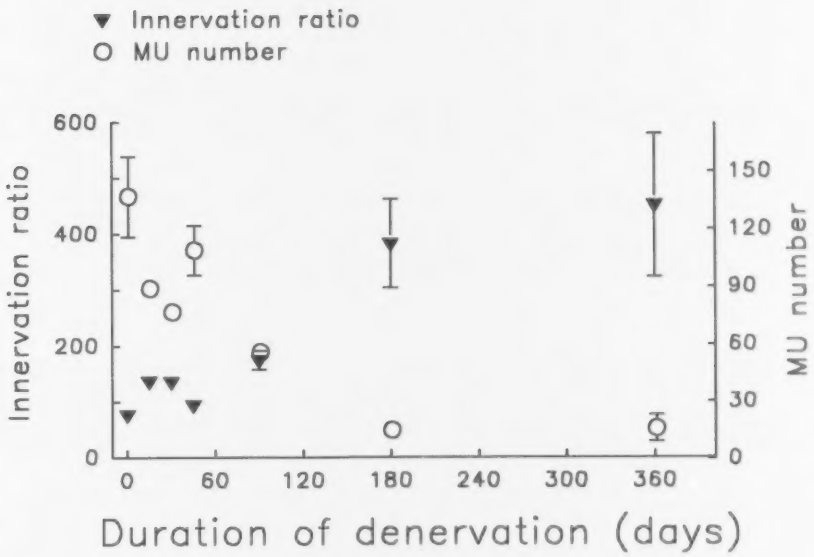

▼ Innervation ratio
○ MU number
Innervation ratio
MU number
600
400
200
0
150
120
90
60
30
0
0
60
120
180
240
300
360
Duration of denervation (days)

Fig. 4.6

Tetanic force in cross-reinnervated (open circles) and contralateral control (filled circles) muscles as a function of the duration of denervation prior to N-N suture.

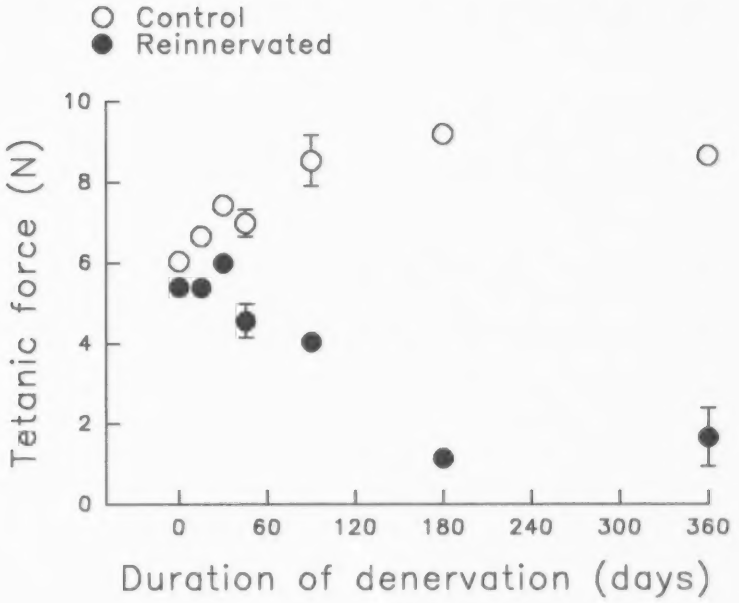

Control
Reinnervated
Tetanic force (N)
10
8
6
4
2
0
0
60
120
180
240
300
360
Duration of denervation (days)

Fig. 4.7

Low and high power photomicrographs of muscle cross-sections in the control (A, B), cross-reinnervated muscle after short-term (30 day) denervation prior to nerve-muscle suture (C, D), and cross reinnervated muscle after long-term (12 month) denervation prior to nerve-muscle suture (E, F). After short-term denervation, muscle size was visibly smaller than the control (C) but the size of muscle fibres is similar to the control (D). After long-term (12 months) denervation prior to N-M suture, both muscle and muscle fibre sizes were much smaller than the control (E, F). The reduced muscle size is the result of reduction in both the number of muscle fibres after short-term denervation and in both the number and size of reinnervated muscle fibres after long-term denervation.

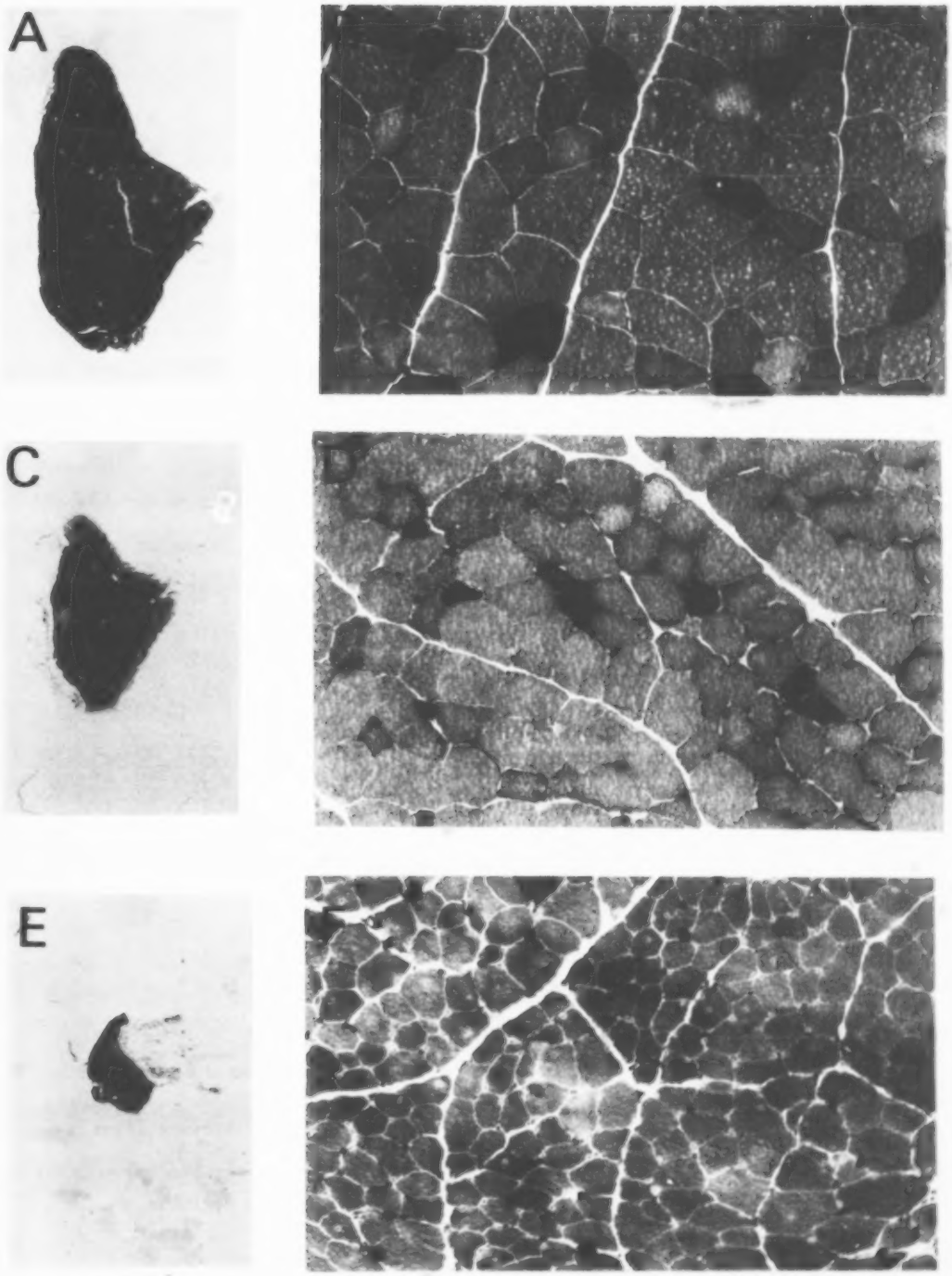

A
C
E
2 mm
50 μm

Fig. 4.8

The effect of the duration of denervation on reinnervated muscle fibre number (A), muscle fibre cross-sectional area (CSA) (B), muscle CSA (C), muscle force (D), MU force (E), and MU number (F) after N-M suture (filled circles) compared with that after N-N suture (open circles). Datum points were expressed as means $\pm$ S.E..

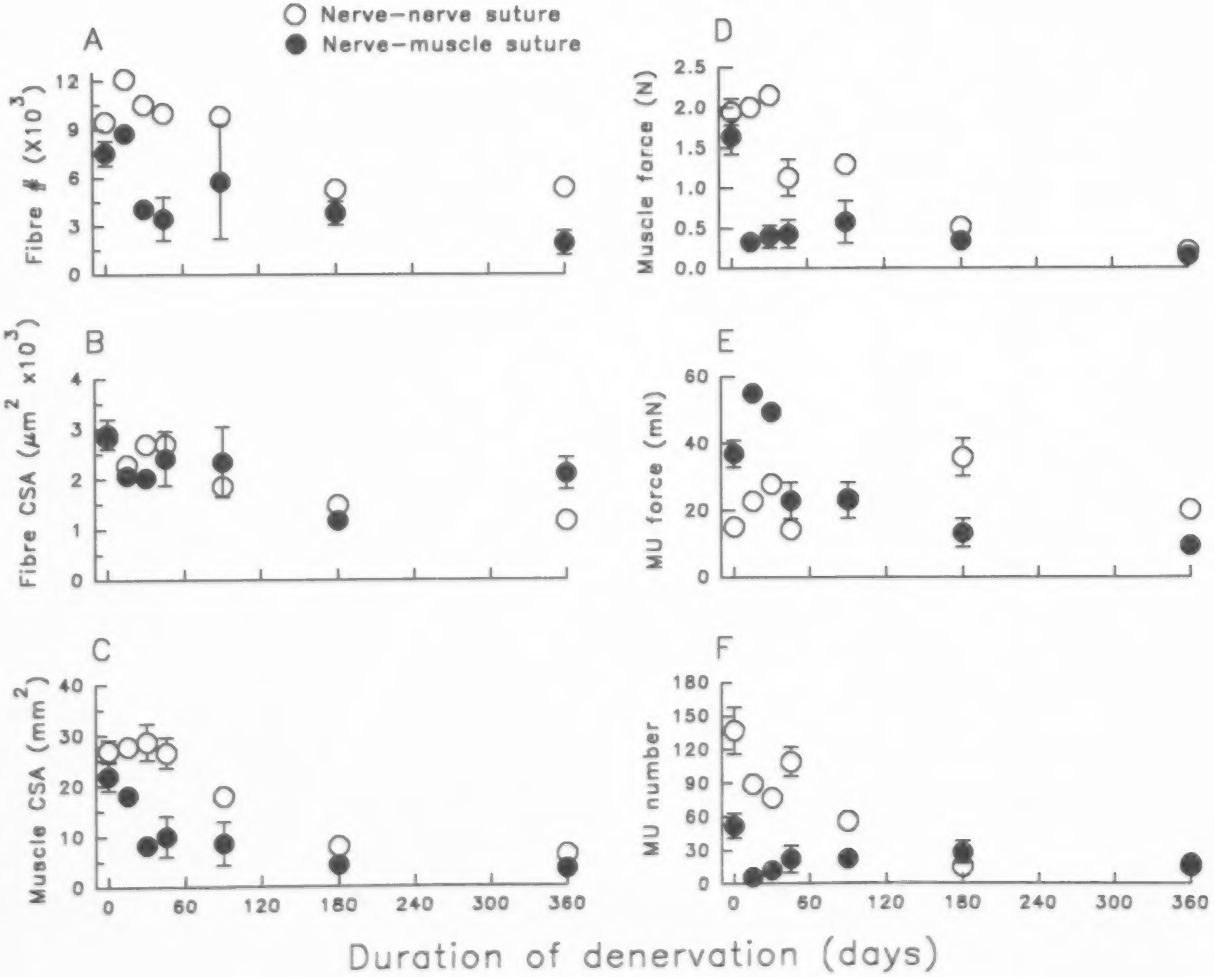

Nerve–nerve suture
Nerve–muscle suture
A
Fibre # (X10^3)
B
Fibre CSA (μm^2 x10^3)
C
Muscle CSA (mm^2)
D
Muscle force (N)
E
MU force (mN)
F
MU number
0 60 120 180 240 300 360
Duration of denervation (days)

Fig. 4.9

Mean (± S.E.) MU number (A), innervation ratio (IR) (B), muscle fibre number (C), muscle fibre cross sectional area (D), and cross-reinnervated muscle force (E) after immediate nerve-nerve, nerve-muscle suture, and after prolonged muscle denervation prior to nerve-nerve, and nerve-muscle suture. Data from reinnervated muscles which had been denervated more 6 months prior to N-N or N-M suture were pooled for comparison with those after immediate nerve repair.

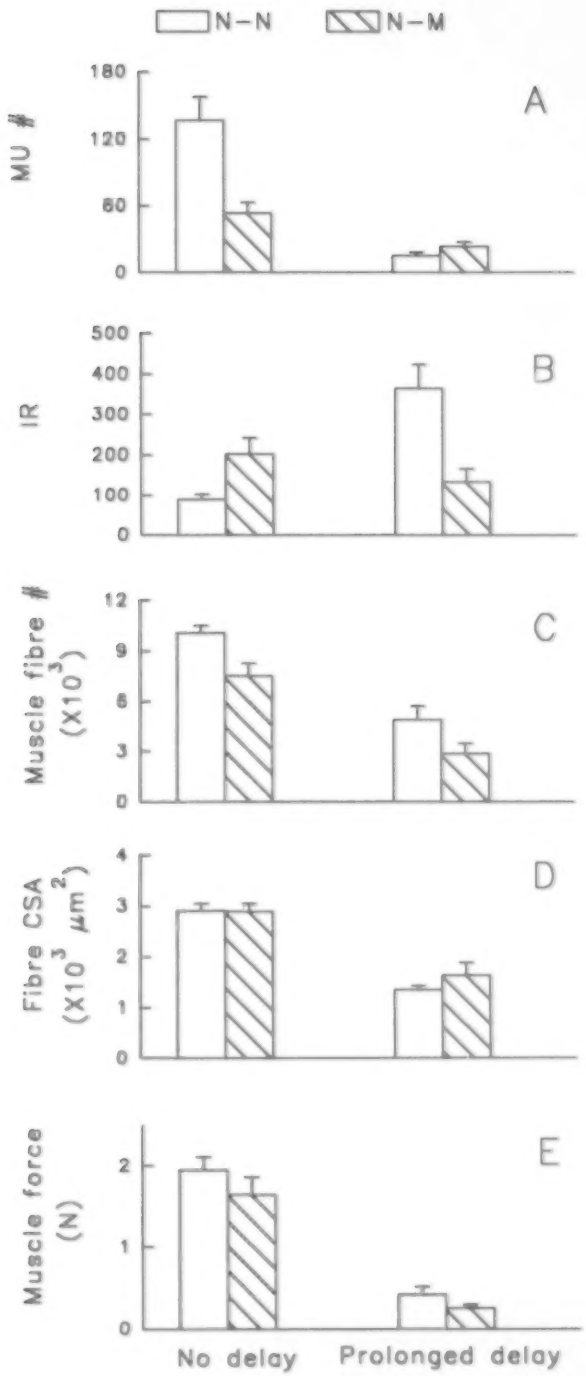
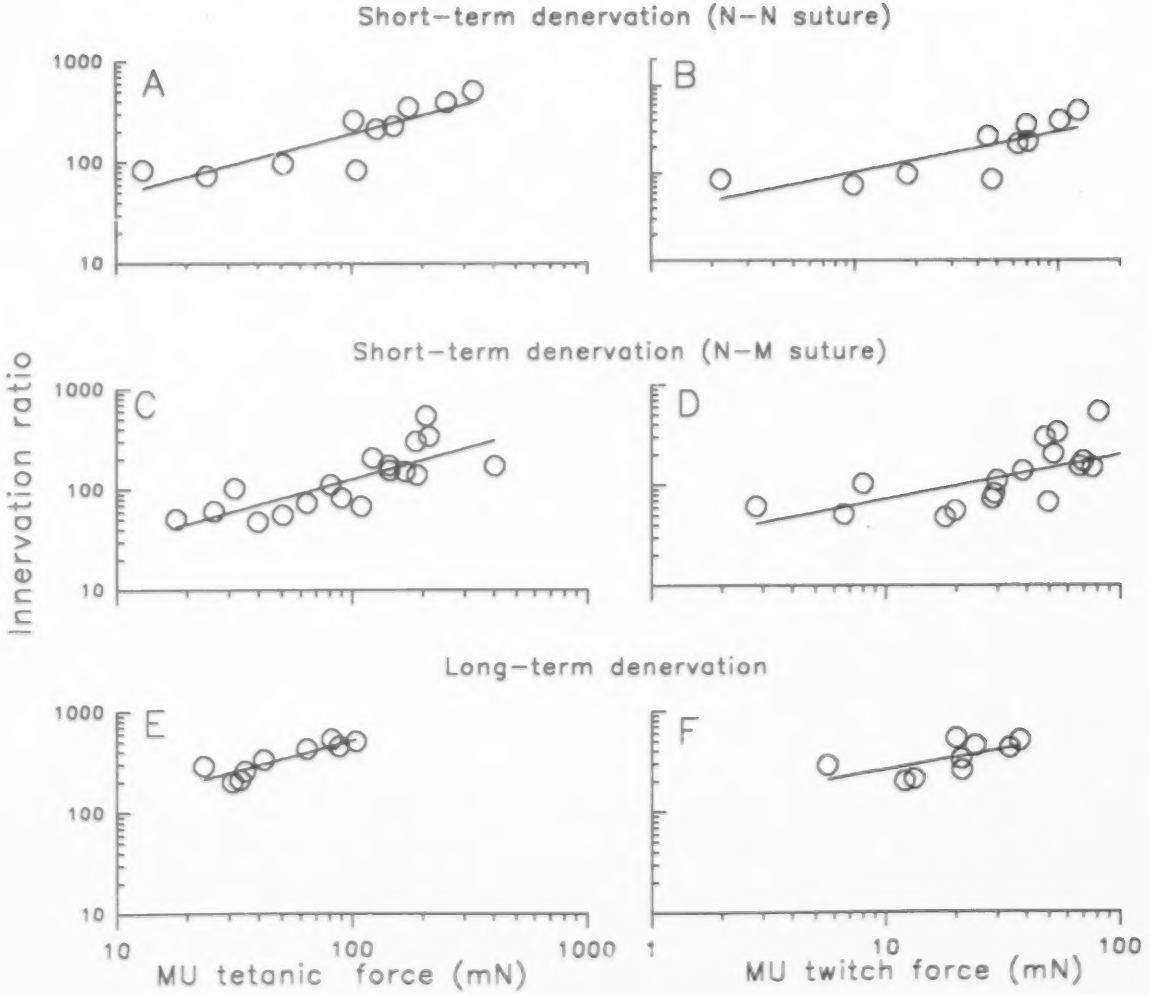

N-N
N-M
MU #
180
120
60
0
A
IR
500
400
300
200
100
0
B
Muscle fibre # (X10^3)
12
9
6
3
0
C
Fibre CSA (X10^3 μm^2)
4
3
2
1
0
D
Muscle force (N)
2
1
0
E
No delay
Prolonged delay

Fig 4.10

Relationship between MU twitch force, tetanic force and innervation ratio in cross-reinnervated muscles after short term denervation (<3 months) prior to N-N suture (A,B), N-M suture (C,D), and long-term denervation (> 6 months) prior nerve repair (E,F). The slopes between tetanic force and IR, and twitch force and IR were 0.59 ± 0.13 and 0.45 ± 0.13 for short-term denervation prior to N-N suture which are significantly different from zero but not significantly different from each other. The slopes ($\pm$S.E.) of the regression lines were 0.64 ± 0.15 (C) and 0.44 ± 0.13 (D) for short-term denervation prior to N-M suture which are not different from that for N-N suture. After long-term muscle denervation prior to nerve repair, the regression lines had similar slopes (0.62 ± 0.13 and 0.43 ± 0.17) to those after short denervation prior to nerve repair. However there were parallel shifts in the regression lines to larger IR values representing the reduced muscle fibre size.


Short–term denervation (N–N suture)
A
B
Short–term denervation (N–M suture)
C
D
Long–term denervation
E
F
Innervation ratio
1000
100
10
1
MU tetanic force (mN)
MU twitch force (mN)

CHAPTER 5

Co-expression of Myosin Heavy Chain Isoforms in Cross-reinnervated Motor Units

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5.I...INTRODUCTION

Since the classical cross-reinnervation experiments of Buller *et al.* (1960), a myriad of studies have confirmed and extended the concept that motoneurones have a strong influence on the metabolic and contractile properties of muscle fibres (Vrbova *et al.*, 1978; Pette and Vrbova, 1985; Pette and Vrbova, 1992; Gordon and Patullo, 1993). This concept has been further tested by using the glycogen depletion technique to study the physiological, morphological and biochemical properties of muscle fibres supplied by a single motor axon (Krnjevic and Miledi, 1958; Edström and Kugelberg, 1968). Normally, muscle fibres of a single motor unit (MU) show homogenous staining intensities for various muscle enzymes (Edström and Kugelberg, 1968; Burke *et al.*, 1971: Nemeth *et al.*, 1981, 1986; Totosy de Zepetnek *et al.*, 1992b). In contrast, muscle fibres of the same histochemical type but belonging to different MUs usually have different intensities of staining to the same enzymes (Edström and Kugelberg, 1968; Burke *et al.*, 1971; Rosser *et al.*, 1991). The discrepancy in the size of muscle fibres of a single MU is much smaller than that of non-MU fibres of the same histochemical type (Martin *et al.*, 1988a,b). Muscle fibres of a normal MU express homogeneous myosin heavy chain (MHC) isoforms (Gauthier *et al.*, 1983; Larsson *et al.*, 1991a; Unguez *et al.*, 1993).

However, accumulating evidence has indicated that neural control over muscle fibre properties may not be complete. The homogeneity of muscle fibres in a single MU has been challenged by findings, obtained by means of quantitative histochemical methods, that

the metabolic enzyme activities of MU muscle fibres are too different for them to be homogeneous (Kugelberg and Lindegren, 1979; Martin *et al.*, 1986a,b). Variance in the size of muscle fibres of a single MU, though often stressed as being smaller than that of non-MU fibres of the same histochemical type, can still be as large as 5 fold. In addition, the size and metabolic enzyme activity among muscle fibres of the same histochemical type vary in different locations in a single muscle suggesting that local factors may also play a role in regulating the properties of muscle fibres (Larsson, 1992; Rosser *et al.*, 1992). Recent studies have shown a small degree of heterogeneity in the expression of MHC isoforms in muscle fibres of single MUs in aged rat tibialis anterior muscles providing additional evidence for incomplete neural control over muscle properties (Larsson *et al.*, 1991b). After self- and cross-reinnervations muscle fibres do not completely lose their original properties and, instead, express different MHC isoforms (Gauthier *et al.*, 1983; Unguez *et al.*, 1993).

Since the majority of muscle fibres in reinnervated MUs in the cat expressed the same MHC isoforms and show a mosaic distribution, Unguez *et al.* (1993) argued that regenerating motor axons reinnervated their original muscle fibre types. However, obvious clumping of MU fibres after self- and cross-reinnervations in rat muscles and homogeneity with respect to their histochemical fibre types (Kugelberg *et al.*, 1970; Totosy de Zepetnek *et al.*, 1992a) do not support this argument. Findings of greater variance in both the MU fibre size (Totosy de Zepetnek *et al.*, 1992b) and metabolic enzyme activity (Sesodia *et al.*, 1994) suggest that conversion of reinnervated muscle

properties may not be complete. Thus, it is still unclear as to whether the phenotype of reinnervated muscle fibres is completely determined by neural innervation.

It is also not known whether neural control over muscle properties is compromised as a result of long-term axotomy and whether long-term denervation influences the response of muscle fibres to neural input. Conceivably there are two ways in which a motoneurone can exert its control over muscle fibre properties: its activity and/or its trophic effects. Both the pattern of activity and the trophic effects of long-term axotomized motoneurones may be different from those of immediately axotomized motoneurones. After long-term muscle denervation, on the other hand, the highly atrophic muscle fibres may differ from freshly denervated muscle fibres in their response to neural regulation. These unknown factors have been analysed in the present study in which a cross nerve suture paradigm in the rat was used to determine the effects of long-term axotomy and long-term muscle denervation on the myosin heavy chain isoform profiles of muscle fibres in single MUs of cross-reinnervated tibialis anterior muscles. Some of these findings have been published in abstract form (Fu *et al.*, 1992).

5.II...MATERIALS AND METHODS

5.II.1...Initial surgery

Forty-four female Sprague Dawley rats (age: 45-60 days; weight: 150-200 g) were used. All surgical procedures were performed under sterile conditions and general anaesthesia induced by i.p. injection of sodium pentobarbital (45 mg/Kg).

In the first group, the tibial (TIB) nerve was sectioned near the ankle and the common peroneal (CP) nerve was sectioned close to muscles in anterior compartment of the right hindlimb. The proximal TIB stump was immediately cross-sutured to the distal CP stump to cross-reinnervate the tibialis anterior (TA) muscle. All muscles innervated by the CP nerve in the anterior compartment except the TA and extensor digitorum longus muscles were carefully removed without damaging blood and nerve supplies to the TA muscle. In some rats the spinal root L5 was sectioned to reduce the number of motor axons in the TIB nerve.

In the second group, the TIB nerve was cut and its regeneration was prevented by ligating and suturing the proximal stump to the normally innervated lateral gastrocnemius muscle. Up to 12 months later, the CP nerve was freshly cut and its proximal stump was ligated and sutured to intact biceps femoris muscle to prevent regeneration. The previously cut TIB proximal stump was refreshed by removing 5 mm from the end. The long-term axotomized TIB nerve was sutured either to the distal stump of the freshly cut

CP nerve or onto the surface of denervated TA muscle. As a result, the freshly denervated TA muscle was now cross-reinnervated by the long-term axotomized TIB nerve.

In the third group, the CP nerve was cut and its proximal stump was ligated and sutured to the biceps femoris muscle to prevent regeneration. As a result, the TA muscle was denervated for up to 12 months. In the second operation, the TIB nerve was freshly cut and its proximal stump was either sutured to the previously cut CP distal stump or onto the surface of the denervated TA muscle. In both cases, the long-term denervated TA muscle was cross-reinnervated by the freshly axotomized TIB nerve.

In all three groups, final experiments were performed at least 6 months after the TIB-CP cross-suture. A single MU in each muscle was isolated and characterised. All unit muscle fibres were depleted of glycogen (described below). At the time of final experiment, the rats in the first group were 9 to 14 months old and 16-26 months old in Groups 2 and 3 in which nerve repair followed long-term axotomy and long-term denervation respectively.

Control data were obtained from age-matched un-operated rats: 6-14 months old rats (Young group) for comparison with Group 1 and 16-26 months old (Old group) for comparison with Groups 2 and 3. Some contralateral control muscles of the experimental rats in Groups 2 and 3 were included in the Old group.

5.II.2...The final experiment

A total of 26 single MUs were isolated and glycogen-depleted in experimental TA muscles and 14 in control TA muscles. For the 14 control MUs, 6 were from 6-13 month old normal rats (Young group) and 8 from both age-matched control muscles and the contralateral control muscles of the experimental rats (Old group). All rats were fed with 5% dextrose 3 to 5 days prior to the final experiment to load muscle glycogen store in order to augment the contrast between glycogen-depleted MU muscle fibres and non-MU fibres. General anaesthesia was induced by i.p. injection of sodium pentobarbital (45 mg/Kg) with 0.1 mg/Kg atropine to reduce tracheal secretion. The left external jugular vein was cannulated for delivering maintenance doses of sodium pentobarbital and atropine. A cannula was inserted into the trachea for mechanical ventilation when needed. Blood volume was maintained by hourly injection of 0.5 ml of 5% dextrose-saline via the venous cannula. ECG was recorded and monitored via an audio monitor for inspection of the depth of general anaesthesia. Vertebrae T13 to L6 were removed to expose the spinal ventral roots L3 to L5.

The TA muscle was isolated by denervating all hip, tail, and other hindlimb muscles. The distal TA tendon was freed and tied with 2-0 silk for attachment to a custom-made force transducer. Two teflon coated silver wires which were used as stimulating electrodes were inserted into the muscle beneath the sciatic nerve with an inter-electrode distance of 10 mm. Bipolar surface patch electrodes were sewn onto the TA muscle fascia for electromyography (EMG) recordings. Both legs were immobilized at

knees and ankles. The left and right TA muscle tendons were attached to custom-made force transducers for isometric force recordings at optimal muscle length. A mineral oil pool was prepared around the spinal cord by stretching the skin around the incision. Dura mater was cut and ventral roots L3 to L6 were isolated with a fine glass rod and gentle suction with a glass pipette. Rectal and muscle temperatures were monitored with probes and maintained at 35°-37°C and 30°-32°C respectively with a heating blanket and an electric bulb.

5.II.3...MU isolation and recordings

Ventral roots were split into small filaments for MU isolation. Force and EMG signals were recorded in response to stimulation of fine filaments containing one axon to the TA muscle. Ten μs pulses were used to stimulate single axons. The criteria for single MU isolation were 1) a single all-or-none twitch contraction and 2) an associated single all-or-none EMG response recorded in response to up to 10 fold threshold voltage. A single MU was selected for further recordings only if the threshold voltage for its activation was lower than 10 volts. These criteria were rigidly met in order to "hold" the unit for the subsequent 1-3 hours of tetanization required for adequate glycogen depletion. Detailed methods for MU isolation have been described in Totosy de Zepetnek *et al.* (1992a).

One MU in each reinnervated or control TA muscle was isolated. Briefly, the following properties were characterized: 1) twitch force and contractile parameters

including contraction time (CT), time to peak contraction (TTP), half-rise time (HRT), and half-fall time (HFT) of twitch contractions, 2) maximum force produced during fused tetanic contractions in response to 100 Hz stimulation, 3) the presence or absence of a decrease ("sag") in the force during un-fused tetanic contractions in response to tetanic trains at intervals of 1.25×contraction time, and 4) fatigability: the decline in the force produced during a two-minute tetanic stimulation (trains of stimuli at 40 Hz with 1 second intervals). MUs were categorized into fast and slow on the basis of presence or absence of sag. As illustrated in Fig. 5.1, fast MUs were subdivided into fast fatigue-resistant (FR), fast fatigue-intermediate (FI) and fast-fatigable (FF) on the basis of fatigue index. MUs with fatigue indices between 1 and 0.75 were classified as FR, between 0.75 and 0.25 as FI, and smaller than 0.25 as FF.

5.II.4...Glycogen depletion

Upon completion of MU characterization, the glycogen store in MU muscle fibres was depleted using the following stimulation protocol. Briefly, short tetanic contractions in a single MU were elicited by trains of stimuli of 5 pulses at 50 Hz delivered every second. When the tetanic force had declined to a steady level, the duty cycle (5 pulse at 50 Hz) was increased progressively from 1 Hz to 2 and 3 Hz and so on. When the force had fallen to less than 10-20% of the original, the rate of stimulation delivery was reduced to 0.2-0.5 Hz to allow the MU muscle fibres to recover their force. This protocol was

repeated up to 12 times until the MU failed to develop 50% of its initial force during a recovery cycle.

5.II.5...Histochemistry

Immediately upon completion of glycogen depletion, the TA muscle was quickly dissected and weighed. Each muscle was cut into three blocks, frozen in isopentane cooled with liquid nitrogen, and stored at -80°C in a freezer for later cryostat sectioning. For each muscle, serial cross-sections each of 10 μm thick were cut and mounted on gelatin-coated slides.

Muscle cross sections were stained for glycogen with Periodic Acid Schiff (PAS) reaction to visualize glycogen-negative muscle fibres which belong to a single MU. Histochemical analysis of myosin ATPase activity was assessed following 1) acid pre-incubation modified from Brooke and Kaiser (1970) and Green *et al.* (1982); and 2) alkaline pre-incubation (modified from Guth & Samaha, 1970). Small modifications was made in the buffers to classify muscle fibres into four types: I, IIA, IIB, and Fint as described in Totosy de Zepetnek *et al.* (1992b). These four types of muscle fibres corresponded with S, FR, FF, and FI MUs respectively.

5.II.6...Myosin heavy chain immunohistochemistry

Five serial 10 μm muscle cross-sections were incubated overnight with each of the following monoclonal primary antibodies 1) BF-F3 (anti-IIB MHC), 2) BF-35 (reacting

with all types of MHCs except type IIX), 3) SC-71 (anti-type IIA MHC), 4) BF-32 (anti-type I and IIA MHC), and 5) BA-D5 (anti-type I MHC). The sections were washed and incubated with a secondary antibody, goat-anti-mouse IgG conjugated with peroxidase, at room temperature. The antibody-antigen binding was visualised by peroxidase reaction with diaminobenzidine (DAB).

5.II.7...Measurements of innervation ratio and fibre size

The number of muscle fibres in a single MU (innervation ratio, IR) was obtained by counting all the glycogen-negative muscle fibres on multiple cross-sections from different levels along the length of a muscle to ensure all MU muscle fibres were included. Cross-sectional area of MU fibres was measured directly using an image analysis system (Java, Jandal Scientific) in which a video camera was connected to the microscope and a personal computer.

5.II.8...Statistics

Student *t* tests were used to compare paired values. One-way analysis of variance (*ANOVA*) was applied for intergroup comparisons. Arithmetic means were calculated and are shown with standard errors (mean$\pm$S.E). For all the statistical analyses, p values of < 0.05 were regarded as significant.

5.III...RESULTS

5.III.1...Correspondence between motor units and fibre types

A total of 14 single MUs in control muscles were classified by their contractile speed, sag, and fatigability into S, FR, FI, and FF (Fig. 5.1). Then, a single MU in each muscle was stimulated repetitively to deplete glycogen content of its muscle fibres for later fibre enumeration, histochemical classification for comparison with the above physiological classification, and immunocytochemical analysis of MHC isoform content. Muscle fibres belonging to single MUs were homogeneous in terms of their relative staining intensity of myosin ATPase after acid and alkaline pre-incubations shown as an example in Fig. 5.2.

As shown in Table 1, all 14 control MUs were classified as F using electrophysiological criteria: 7 FFs, 4 FIs, and 3 FRs. Consistent with previous observations in the same muscle the MUs were ranked from FR-FI-FF in accordance with tetanic force and IR (Totosy de Zepetnek *et al.*, 1992a). Using acid-stable myosin ATPase, all 7 FFs were found to contain the corresponding type IIB muscle fibres. In 4 FIs, 1 contained Fint muscle fibres but the other 3 contained IIB fibres. Of the 3 FRs, 2 contained IIA muscle fibres and the remaining 1 contained Fint fibres. Thus, in 4 out of the total 14 MUs (28%), physiological classification did not agree with histochemical classification. Similar discrepancy between physiological and histochemical classifications

has been reported for both rat and cat TA muscles (Dum and Kennedy, 1980; Totosy de Zepetnek *et al.*, 1992b).

A total of 26 MUs were characterized in cross-reinnervated muscles at least 6 months after nerve repair: 7 from muscles after immediate nerve repair, 10 from muscle reinnervated by long-term axotomized TIB neurons, and 9 from reinnervated muscles which were denervated for prolonged time prior to nerve repair. All 26 MUs were classified as F using electrophysiological criteria: 10 FF, 11 FI and 5 FR MUs. Likr control MUs, reinnervated MUs were again ranked in order of FF->FI->FR according to tetanic force and IR (Table 2 to 4). Also similar to the control, all MU muscle fibres were of the same histochemical type on the basis of acid-stable and labile myosin ATPase staining. But physiological and histochemical classifications failed to correlate in 27% of the reinnervated MUs (see Fig 5.1). In 10 FF units, 5 contained IIB fibres and the other 4, Fint fibres. In 11 FI unit, 9 were composed of Fint fibres, 1 of IIB fibres and 1 of IIA fibres. Four out of the 5 FR MUs contained IIA fibres and the other one, S fibres.

MUs will be referred by their histochemical types below, namely type IIB, IIA, Fint, and I.

5.III.2...Myosin heavy chain expression in MU muscle fibres

5.III.2.a...Control muscles

Of the 14 MUs processed for MHC immunohistochemistry, 6 were from the Young group (6-13 months old) and the other 8 MUs were from the Old group (9-26 months old).

In the Young group, 4 out of 6 were classified as IIB, one as Fint, and one as IIA on the basis of histochemistry (Table 1). Muscle fibres in all the 6 MUs expressed corresponding MHC isoforms. All muscle fibres in IIB MUs contained IIB MHC: positive immunoreactivity was observed for 1) BF-F3 antibody and 2) BF-35 antibody (reacting with all types of MHCs except type IIX). The muscle fibres did not react to SC-71 (anti-IIa) or BF-32 (anti-type I and IIa), and BA-D5 (anti-type I). All muscle fibres in the Fint MU expressed IIX MHC: with not reaction to antibodies against all MHC isoforms except IIX, against IIB, IIA, and I MHCs. Muscle fibres in the one IIA MU expressed only type IIA MHC and showed positive immunoreactivity to antibodies against IIA (SC-71), both IIA and I (BF-32), and all MHC isoforms except IIX (BF-35), but negative reaction to antibody against type IIB (BF-F3) and I (BA-D5).

The total of 8 MUs from the Old Group included 6 IIB MUs, one Fint and one IIA. In contrast to the Young group, 4 out of 8 MUs showed a small degree of heterogeneity in the expression of MHC isoforms. Amongst the 6 IIB MUs, 3 contained muscle fibres which expressed type IIB MHC. The majority of the muscle fibres in the other 3 MUs expressed type IIB MHCs: 1- 6% of muscle fibres did not react to BF-35 antibody (against all MHCs except IIX) and therefore were considered to express MHC IIX (Fig. 5.2). In the one FI MU, 99% of the muscle fibres expressed IIX MHC (negative immunoreactivity to BF-35) with the remaining 1% expressing IIB MHC. All fibres in the IIA MU expressed MHC IIA isoform.

In spite of the heterogeneity in MHC expression in 50% of the MUs, the majority of MU muscle fibres expressed the MHC isoforms which corresponded to the histochemical fibre type. Similar results have been reported in aged albino rats (Larsson *et al.*, 1991b).

5.III.2.b...Reinnervated TA muscles after TIB-CP cross suture

MUs in reinnervated muscles contained significantly more muscle fibres than the control MUs ($p < 0.01$) (*cf* IR in Tables 1 & 2 to 4). MU tetanic force was also higher than normal except in the case of prolonged muscle denervation prior to nerve repair where reinnervated muscle fibres did not completely recover from denervation atrophy (Chapters 2, 3, & 4). Fibres within a single MU were more tightly clumped coincidental with a trend of grouping of the same of muscle fibre type.

MUs were grouped according to immediate nerve repair, prolonged axotomy, and prolonged denervation prior to nerve repair.

Immediate nerve cross-suture. The 7 MUs studied included 1 IIB, 3 Fints and 3 IIAs. Although MU muscle fibres were homogeneous with respect to histochemical type, only 3 IIA MUs of 7 MUs, the IIA MUs, expressed one MHC isoform. Muscle fibres in the remaining 4 MUs (1 IIB and 3 Fint) expressed either of the two MHC isoforms. The majority of muscle fibres in the IIB MU expressed IIB MHC with a small proportion (up to 11%) expressing IIX MHC (Fig. 5.3). Two Fint MUs were composed of fibres expressing IIX MHC (56% and 59%) with the rest expressing IIB MHC. The majority

of muscle fibres in the other Fint MU expressed IIA MHC (68%) with the rest expressing IIX MHC (32%).

Muscle fibres all 7 MUs isolated from age-matched control rats (the Young group) expressed only one MHC isoforms.

Prolonged motoneurone axotomy. Of the 10 MUs examined, there were 3 IIB, 6 Fint and 1 S. The composition of MHCs in these MUs were very similar to those in reinnervated muscles after immediate nerve cross-suture. In the only Type I MU studied, all muscle fibres expressed Type I MHC. In the remaining 9 MUs (3 IIB and 6 Fint), MU muscle fibres expressed two types of MHC isoforms.

All IIB and Fint MUs contained muscle fibres which expressed one of the 2 MHC isoforms: MU muscle fibres were either IIB and IIX or IIX and IIA. In the 3 IIB units, most (73% and 92%) fibres expressed IIB MHC with the rest of muscle fibres expressing MHC IIX (Fig. 5.4). In the Fint MUs, the majority of muscle fibres could express any of the 3 fast MHC isoforms, IIB, IIX, or IIA.

Significantly more MUs in reinnervated muscles contained muscle fibres expressing different MHC isoforms than MUs in age-matched control muscles (the Old group) ($p < 0.01$). Therefore the observed heterogeneity of MHC expression cannot be solely attributed to ageing.

Prolonged muscle denervation. A total of 9 MUs were studied including 3 IIB, 4 Fint and 2 IIA MUs. MHC composition in muscle fibres of reinnervated MUs was similar to that seen after both immediate nerve repair and prolonged axotomy in the sense

that muscle fibres in IIB and Fint MUs expressed one of two MHC isoforms. The number of MUs with more than one MHC isoforms was significantly greater than age-matched control (the Old group) ($p < 0.01$). In the two IIA MUs, all muscle fibres expressed IIA MHCs. In two IIB MUs, the majority (70% and 96%) of the muscle fibres expressed MHC IIB with the rest expressing IIX MHC. In the other IIB unit, however, the majority (70%) of muscle fibres expressed IIX MHC with the rest expressing IIB MHC. In three Fint MUs, most muscle fibres expressed IIX MHC (53%-95%) with the rest expressing IIB in two units and IIA in the other. In the other Fint unit, the majority of muscle fibres (75%) expressed IIA with the rest expressing IIX MHCs.

5.III.3...Muscle fibre size in single MUs

In the control TA muscles, muscle fibres innervated by single motor axons were not identical in size. Muscle fibre cross-sectional area varied over a 1 - 6.6 fold range, an average of 3.63 ± 0.51 fold (Fig. 5.6). The size of muscle fibres tended to increase in the order of IIA < Fint < IIB.

After immediate nerve cross-suture, the range in muscle fibre size was 3.2 - 8 fold with 7 MUs, an average range of 5.35 ± 1.64. The range in the reinnervated MUs was significantly larger than in normal MUs. The range was even higher in reinnervated muscles after prolonged axotomy or prolonged denervation prior to nerve repair (4.5-12.4 with a mean of 8.82 ± 0.79, and 2.3-36.5 with a mean of 8.52 ± 0.79 respectively).

5.IV...DISCUSSION

The present study shows that muscle fibres of long-term reinnervated single MUs often express two MHC isoforms and are more variable in size than the control. These findings indicate that muscle properties are not entirely determined by neural innervation. On the other hand, homogeneous S and FR units and limited heterogeneity in FI and FF units show that adult muscle phenotype is strongly influenced by the motor nerve. The pattern of MHC expression in muscle fibres of reinnervated MUs was not affected by either long-term axotomy or long-term muscle denervation prior to the nerve repair.

5.IV.1...Incomplete conversion of muscle phenotype after reinnervation

In normal rat hindlimb muscles, fibres of different histochemical type intermingle to form the characteristic mosaic distribution (Kapati and Engel, 1968). However, after either self- or cross-reinnervation, muscle fibres of the same histochemical type tend to clump (Kugelberg *et al.*, 1970; Gordon *et al.*, 1991; Totosy de Zepetnek *et al.*, 1992a). Type grouping indicates that regenerating axons neither reinnervate the original muscle fibres nor muscle fibres of the original type. Instead, regenerating axons supply muscle fibres which formerly belong to different MUs. This argument is supported by the finding that reinnervated MUs initially contain more than one fibre type (Warszarski *et al.*, 1975). With time after reinnervation, MUs become homogeneous in muscle fibre composition (Kugelberg *et al.*, 1970; Gordon *et al.*, 1988; Totosy de Zepetnek *et al.*, 1992b). In the

present study where TA muscles were examined at least 6 months after cross-reinnervation by the TIB nerve, all MU fibres were of the same histochemical type. The restoration of muscle fibre homogeneity indicates that the histochemical profile of muscle fibres is strongly influenced by the reinnervating axon. Yet, a large majority of reinnervated MUs contained more than two types of muscle fibres with respect to their MHC composition. In the one S MU and 5 FR MUs, all fibres expressed the corresponding type I MHC and IIA MHC respectively. However, FI and FF MUs always contained fibres which expressed either IIA or IIB MHC isoforms together with IIX MHC isoform. Evidently, the pH sensitivity of myosin ATPase which permits the resolution of 4 fibre types is not sufficient to detect the existence of more than one MHC isoform in MU fibres in the rat. Similarly reinnervated muscles in the cat are homogeneous in histochemical type despite expression of more than one MHC isoform (Gauthier *et al.*, 1983; but *cf* Unguez *et al.*, 1993).

Some muscle fibres in reinnervated cat MUs co-expressed more than 2 MHC isoforms (Gauthier *et al.*, 1983). It is likely that a single muscle fibre in our cross-reinnervated FI and FF MUs also expressed two type of MHC isoforms. However, because the IIX MHC was identified using antibody against all MHC isoforms except IIX, it was not possible to detect hybrids fibres which might have included IIB and IIX, or IIX and IIA MHC isoforms.

In addition to MHC heterogeneity, MUs in the cross-reinnervated muscles in the present study also showed larger than normal inter-fibre differences in oxidative and

glycolytic enzyme activity (Sesodia *et al.*, 1994) and a greater than normal variability in size. Interestingly, S and FR units showed more heterogeneity with respect to size and metabolic enzyme activity even though these MUs did not co-express different MHC isoforms.

5.IV.2...Factors controlling reinnervated muscle phenotypes

The demonstrated heterogeneity of reinnervated MU fibres argues against the view that muscle properties are completely determined by the nerve and the muscle activity that it mediates (Buller *et al.*, 1960; Vrbova *et al.*, 1978). Several other observations point to the same conclusion. For instance, after cross-reinnervation by a fast nerve, the soleus muscles in both the cat and rat were not completely transformed with all fibres retaining their high oxidative capacity (Chan *et al.*, 1982; Gillespie *et al.*, 1987). In addition, a much higher than expected proportion of slow MUs was maintained in the cross-reinnervated soleus muscle (Chan *et al.*, 1982; Foehring and Munson, 1990). Cross-reinnervated fast muscles continued to show "sag" despite slower contraction (Dum *et al.*, 1985). Chronic electrical stimulation of fast twitch muscles with the pattern of activity similar to that of slow motoneurones induces a significant increase in the number of hybrid fibres which co-express two or more MHC isoforms (Staron *et al.*, 1987; Maier *et al.*, 1988; Termin *et al.*, 1990) and fails to convert type II fibres into type I in rats (Termin *et al.*, 1989; Ausoni *et al.*, 1990). In addition, "fast" patterns of stimulation did not convert type I fibres in the soleus muscle to type IIB (Gorza *et al.*, 1988).

There are several possible explanations for the incomplete conversion by reinnervation. One is that insufficient time elapsed to allow for complete transformation. However this is unlikely in the present study because the same heterogeneity was seen after 17 months of reinnervation as after 6 months. Second, heterogeneity is also unlikely to be completely age-related because heterogeneity was very low in age-matched control muscles. Third, it is possible that muscle fibres within each reinnervated MU were not equally activated. Initial low synaptic efficacy at newly innervated neuromuscular junctions may fail to transmit the same pattern and amount of activity to all the muscle fibres (Tonge, 1974; Dennis & Miledi, 1974). However, recovery of high synaptic efficacy after long-term reinnervation indicates that the high safety factor of neuromuscular transmission may be restored (Labovitz and Robbins, 1983). Fourth, clonally derived differences in muscle fibre phenotype may be partially retained in the adult despite novel reinnervation. This has been the suggested explanation for 1) the developmental expression of phenotype even in the absence of innervation (Butler *et al.*, 1982; Miller & Stockdale, 1986, 1987; Condon *et al.*, 1990), 2) incomplete conversion of adult rat muscle by chronic stimulation (Gorza *et al.*, 1988; Ausoni *et al.*, 1990), and 3) normal regionalization of fibre types is partially restored after reinnervation (Parry and Wilkinson, 1991). However since complete conversion of rat muscle properties may occur after chronic stimulation if the hyperthyroid status of the rat is corrected (Kirschbaum *et al.*, 1990) this explanation is unlikely to fully account for the observed MU heterogeneity in reinnervated muscles. Fifth, the co-expression of different MHC isoforms in reinnervated

Fint and FF MUs could be linked to muscle architecture. Fast fibres are normally located in superficial regions of the muscle where their length, load and position remain unchanged despite reinnervation. Differences in the mechanical load experienced by fibres in different regions may contribute to their phenotypic expression. This is supported by findings that expression of fast MHC isoforms was induced in soleus muscle fibres by reducing muscle load (Lowrie *et al.*, 1989; Gupta and Zak, 1992).

In summary, the phenotype of cross-reinnervated muscle fibres is not only regulated by neural activity but also by other factors such as intrinsic regulation, hormones and those associated with the muscle architecture. The coexistence of IIB or IIA fibres with IIX fibres but not with each other in single reinnervated MUs is consistent with the suggested order of transformation by neural activity, namely IIB-> IIX -> IIA -> I (Pette and Vrbova, 1992).

5.IV.3...Reinnervated MU heterogeneity after delayed nerve repair

The extent of heterogeneity in reinnervated MUs was the same after immediate nerve repair as after prolonged axotomy. Even when motoneurones remained axotomized for 12 months prior to nerve repair, the extent of heterogeneity did not increase. This was true even though axotomy significantly reduced the ability of the axons to successfully reinnervate muscle (Fu and Gordon, 1994). Thus, long-term axotomy does not reduce the capacity of the motoneurones to regulate muscle properties.

Motoneurone axotomy induces a series of changes which appear to convert the axotomized neuron from a transmitting to a growth mode (Gordon, 1983). For example growth associated proteins including GAP-43 and T-α tubulin are expressed an increasing amount while neurofilaments and transmitter-related proteins are down-regulated (Miller *et al.*, 1989; McQuarrie and Lasek, 1989). Down-regulation of neurofilament protein is associated with a decline in axonal size (Hoffman *et al.*, 1987; Gordon *et al.*, 1991). In long-term axotomized motoneurones, cell bodies and axons become severely atrophic and express very low levels of tubulin (Cassar *et al.*, 1991; Gordon *et al.*, 1991). Nevertheless, the motoneurones respond to a second axotomy with an upregulation of tubulin and GAP 43 (Cassar and Tetzlaff, 1991) and many axons regenerate to make functional connections with muscle (Fu and Gordon, 1994b). Reformation of functional connections reverses the changes associated with axotomy (Gordon, 1983; Titmus and Faber, 1990) although recovery of electrical properties is slow (Gordon and Stein, 1982; Foehring *et al.*, 1986). Whether complete recovery is compromised by long-term axotomy is not clear. Nevertheless, regenerated motor axons faithfully conduct action potentials and activate muscle fibres in reinnervated muscles (Fu and Gordon, 1994). For example, reinnervated MU muscle fibres contracted in response to single stimulus pulses and to tetanic trains at frequencies of 10-100 Hz. During repetitive stimulation for glycogen depletion in the present study, the MU fibres contracted continuously for up to 4 hours required for glycogen-depletion. Thus it is likely that the nerve-mediated activity of

reinnervated MUs controls muscle phenotype to the same extent as after immediate nerve repair.

The MHC composition in reinnervated muscle fibres which had been denervated for prolonged time prior to nerve repair was also not different from that after immediate nerve repair. Even though, after long-term denervation, regenerating axons supply highly atrophic muscle fibres as well as myotubes derived from satellite cell division and maturation (Anzil & Wernig, 1989; Schmalbruch *et al.*, 1991), heterogeneity was not higher than after reinnervation of freshly denervated muscles. Thus, regenerating axons control the phenotype of highly atrophic muscle fibres and regenerating muscle fibres to the same extent as freshly denervated muscle fibres. Since reinnervated MUs probably include both the original and regenerated muscle fibres, our findings also show that nerves have a strong influence in determining the phenotype of immature as well as mature atrophic muscle fibres. For example, the long-term reinnervated FR MU was similarly homogeneous in MHC composition as after immediate nerve repair. These findings are consistent with neural determination of regenerated muscle phenotype in adults (Gordon and Vrbova, 1975; Gordon *et al.*, 1977).

It is likely that neural influence is mediated by activity as for the freshly denervated muscle. Even in the absence of the nerve, electrical stimulation of long-term denervated muscles was able to reorganise myofilaments and permit some recovery of fibre size (Al Amood *et al.*, 1991; Schmalbruch *et al.*, 1991).

5.IV.4...Conclusions

Incomplete conversion of muscle phenotype in reinnervated single FI and FF MUs show that factors in addition to nerve-mediated activity contribute to the regulation of muscle phenotype. These factors include intrinsic regulation, hormones, and mechanical factors associated with muscle architecture. The coexistence of not more than 2 isoforms in reinnervated FI and FF MUs and the homogeneity of S and FR MUs supports the major role of neural activity in determining muscle fibre phenotype. This control does not diminish with long-term axotomy prior to muscle reinnervation. Neural regulation is also equally effective in long-term denervated muscles which undergo extensive atrophy and where MUs are composed of regenerated muscle fibres.

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Table 5.1. Control motor units: myosin heavy chain profiles of motor unit (MU) muscle fibres

MU	Tetanic force (mN)	IR	Physiol. type	Histochem. type	MHC composition (%) IIB	IIX	IIA	I
Ycon1	30	58	FR	IIA	0	0	100	0
Ycon2	49	85	FR	IIC	0	100	0	0
Ycon3	122	190	FF	IIB	100	0	0	0
Ycon4	86	94	FF	IIB	100	0	0	0
Ycon5	80	124	FI	IIB	100	0	0	0
Ycon6	37	92	FI	IIB	100	0	0	0
mean	81	125			100	0	0	0
SE	15	20			0	0	0	0
Ocon1	33	67	FR	IIA	0	0	100	0
Ocon2	31	85	FI	IIC	1	99	0	0
Ocon3	128	94	FI	IIB	100	0	0	0
Ocon4	111	124	FF	IIB	95	5	0	0
Ocon5	144	203	FF	IIB	100	0	0	0
Ocon6	89	153	FF	IIB	94	6	0	0
Ocon7	51	92	FF	IIB	98	2	0	0
Ocon8	91	100	FF	IIB	100	0	0	0
mean	103	128			98	2	0	0
SE	12	16			1	1	0	0

Ycon = young control rat; Ocon = old control rat; Physiol. = Physiological; Histochem. = Histochemical.

Table 5.2. Reinnervated motor units after immediate nerve suture: myosin heavy chain profiles of motor unit (MU) muscle fibres

MU	Tetanic force (mN)	IR	Physiol. type	Histochem. type	MHC composition (%) IIB	IIX	IIA	I
1	207	259	IIA	IIA	0	0	100	0
2	79	231	IIA	IIA	0	0	100	0
3	32	307	IIA	IIA	0	0	100	0
mean	106	266			0	0	100	0
SE	43	18			0	0	0	0
4	155	382	FF	IIC	41	59	0	0
5	173	296	FI	IIC	44	56	0	0
6	317	207	FI	IIC	0	68	32	0
mean	215	295			28	61	11	0
SE	42	41			12	3	9	0
7	329	511	FF	IIB	89	11	0	0

Physiol. = Physiological; Histochem. = Histochemical.

Table 5.3. Reinnervated motor units after prolonged axotomy: myosin heavy chain profiles of motor unit (MU) muscle fibres

					MHC composition (%)			
MU	Tetanic force (mN)	IR	Physiol. type	Histochem. type	IIB	IIX	IIA	I
1	62	60	IIA	I	0	0	0	100
2	82	118	FI	IIC	78	22	0	0
3	138	292	FI	IIC	0	95	5	0
4	40	48	FF	IIC	13	87	0	0
5	168	150	FF	IIC	45	55	0	0
6	81	112	FI	IIC	0	27	73	0
7	145	154	FI	IIC	81	19	0	0
mean	109	146			36	51	13.0	0.0
SE	18	30			14	13	11.0	0.0
8	249	347	FF	IIB	73	27	0	0
9	230	173	FF	IIB	92	9	0	0
10	615	553	FF	IIB	89	11	0	0
mean	365	358			85	16	0.0	0.0
SE	102	90			5	5	0.0	0.0

Physiol. = Physiological; Histochem. = Histochemical.

Table 5.4. Reinnervated motor units after prolonged denervation: myosin heavy chain profiles of motor unit (MU) muscle fibres

MU	Tetanic force (mN)	IR	Physiol. type	Histochem. type	MHC composition (%) IIB	IIX	IIA	I
1	31	300	FR	IIA	0	0	100	0
2	53	116	FR	IIA	0	0	100	0
mean	42	208			0	0	100	0
SE	8	65			0	0	0	0
3	128	213	FI	IIC	5	95	0	0
4	24	73	FI	IIC	0	53	47	0
5	151	228	FF	IIC	16	84	0	0
6	207	541	FI	IIC	0	25	75	0
mean	128	264			5	64	31	0
SE	33	86			3	14	16	0
7	88	449	FF	IIB	30	70	0	0
8	33	213	FI	IIB	70	30	0	0
9	253	396	FF	IIB	96	4	0	0
mean	125	353			65	35	0	0
SE	54	58			16	16	0	0

Physiol. = Physiological; Histochem. = Histochemical.

Fig. 5.1

Physiological classification of motor units in normal (A) and reinnervated (B) TA muscles and its correspondence with histochemical muscle fibre types. Horizontal lines subdivide fast MUs according to fatigue index (FR > 0.75 < FI > 0.25 > FF). FR, FI, and FF MUs contained IIA, Fint, IIB muscle fibres (open circles) except for 4 out of 14 MUs in control muscles and 6 out of 26 in reinnervated muscles (filled circles).

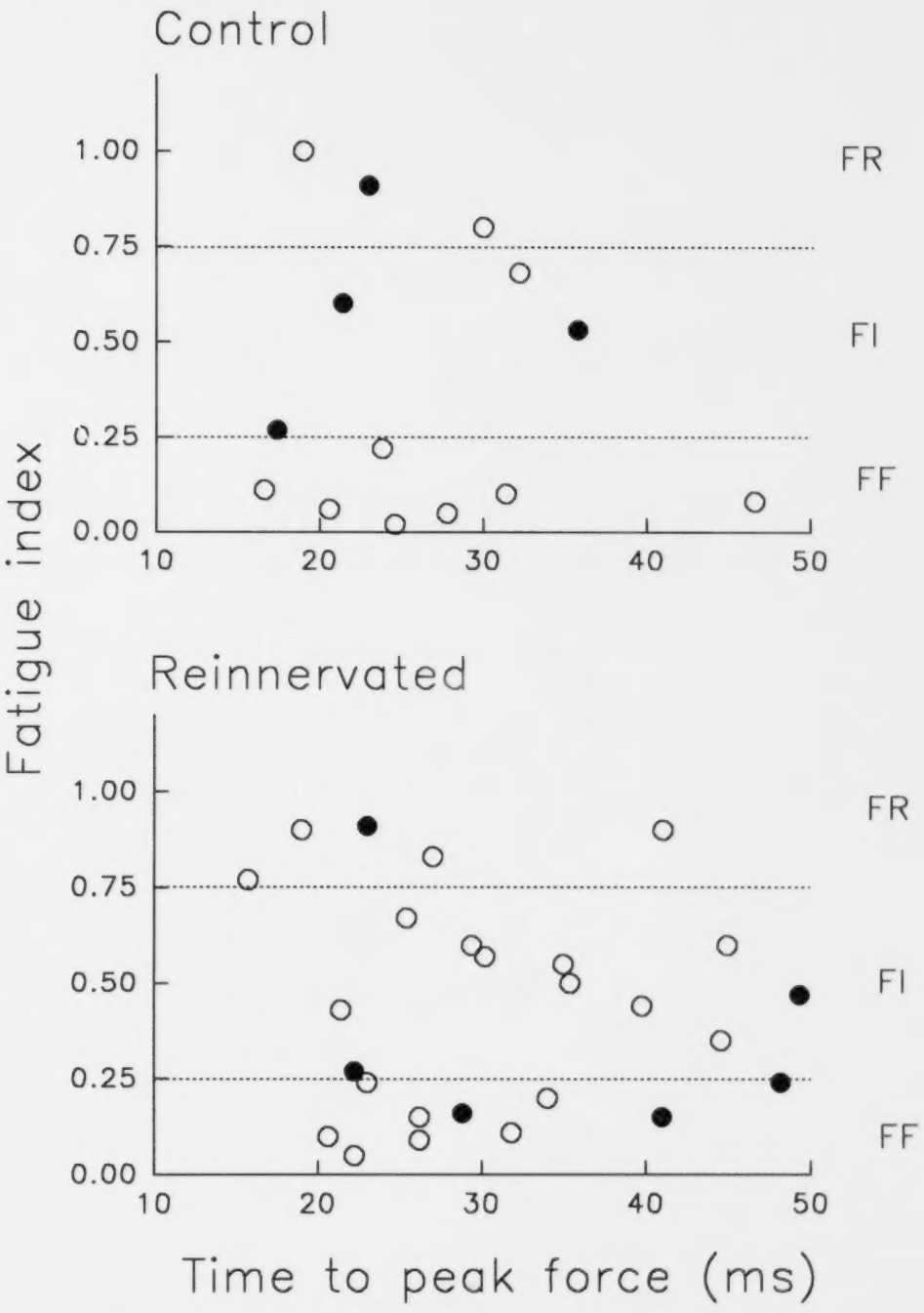

Control
Reinnervated
Fatigue index
1.00
0.75
0.50
0.25
0.00
10
20
30
40
50
FR
FI
FF
Time to peak force (ms)

Fig. 5.2

Serial sections of a TA muscle from a 20-month-old control rat (Old group) stained for periodic acid-Schiff (PAS) reaction (A), mATPase with acid pre-incubation (B), and reacted with monoclonal antibodies BF-F3 (against IIB MHC) (C), BF-35 (against all MHC isoforms except IIX) (D), BF-32 (against IIA and MHCs) (E), and BA-D5 (against I MHC) (F). Two of 5 glycogen depleted MU fibres were identified in all the sections for comparison. Staining for mATPase identified MU muscle fibres as IIB. However, they did not contained uniform MHC isoforms. Fibre (a) reacted with BF-F3 and BF-32 antibodies and therefore expressed IIB MHC. Fibre (b) did not react with any of the antibodies and therefore expressed IIX MHC.

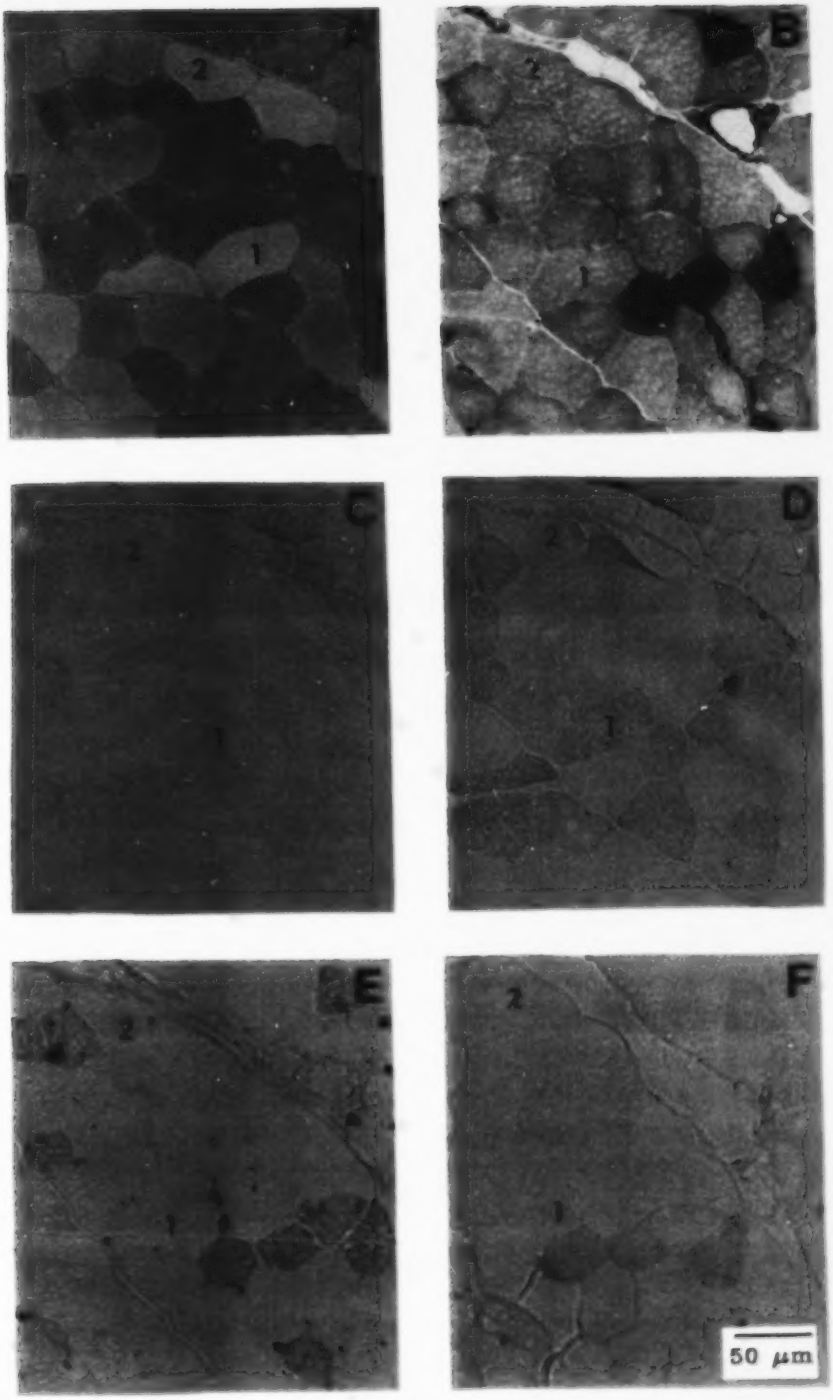

B
C
D
E
F
50 μm

Fig. 5.3

Serial cross sections of a cross-reinnervated TA muscle after immediate nerve repair stained for periodic acid-Schiff (PAS) reaction (A), mATPase with acid pre-incubation (B), and reacted with monoclonal antibodies BF-F3 (against IIB MHC) (C), BF-35 (against all MHCs but IIX) (D), SC-71 (against IIA MHC) (E), and BA-D5 (against I MHC) (F). MU muscle fibres shown in (A) highly clumped and was stained uniformly for mATPase as IIA (B). All MU fibres reacted with antibody BF-35, SC-71 but not with BF-F3 and BA-D5 and therefore expressed IIA MHC. Two of the glycogen-depleted MU muscle fibres were identified in all the sections for comparison.

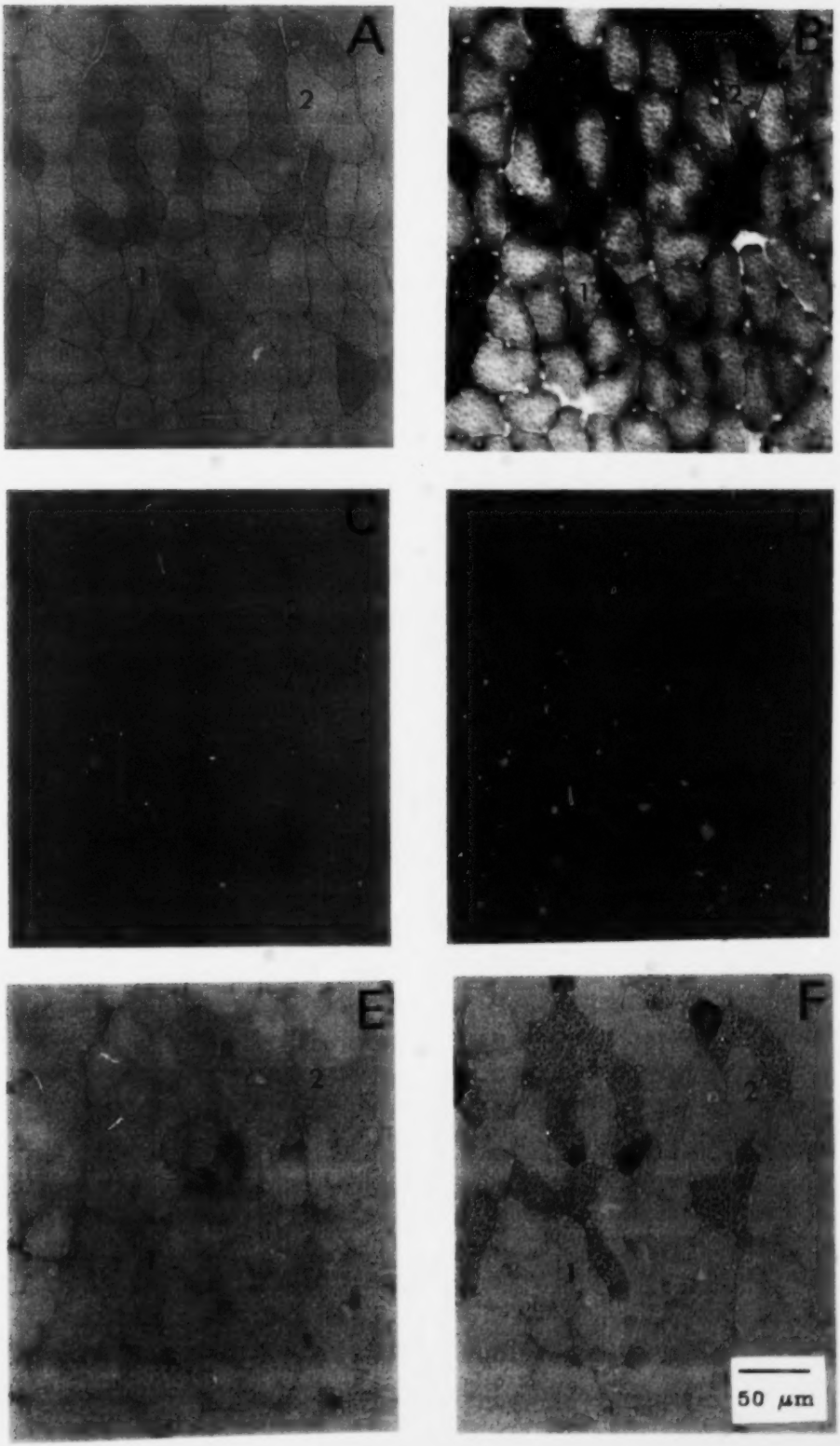

A
2
1
B
2
1
C
E
2
F
2
1
50 µm

Fig. 5.4

Serial cross sections from a cross-reinnervated muscle after prolonged axotomy prior to nerve repair stained for periodic acid-Schiff (PAS) reaction, mATPase with acid pre-incubation, and reacted with monoclonal antibodies BF-F3 (against IIB MHC) (C), BF-35 (against all MHCs but IIX) (D), BF-32 (against IIA and I MHCs) (E), and BA-D5 (against I MHC) (F). The glycogen-depleted MU fibres shown in (A) highly clumped and has stained uniformly for mATPase as type Fint (B). However, they did not contained uniform MHCs. Two MU muscle fibres were identified in all sections for comparison. Fibre (a) was stained by BF-F3 and SC-75 and therefore expressed IIB MHC. Fibre (b) was not stained by any of the antibodies and therefore expressed IIX MHC.

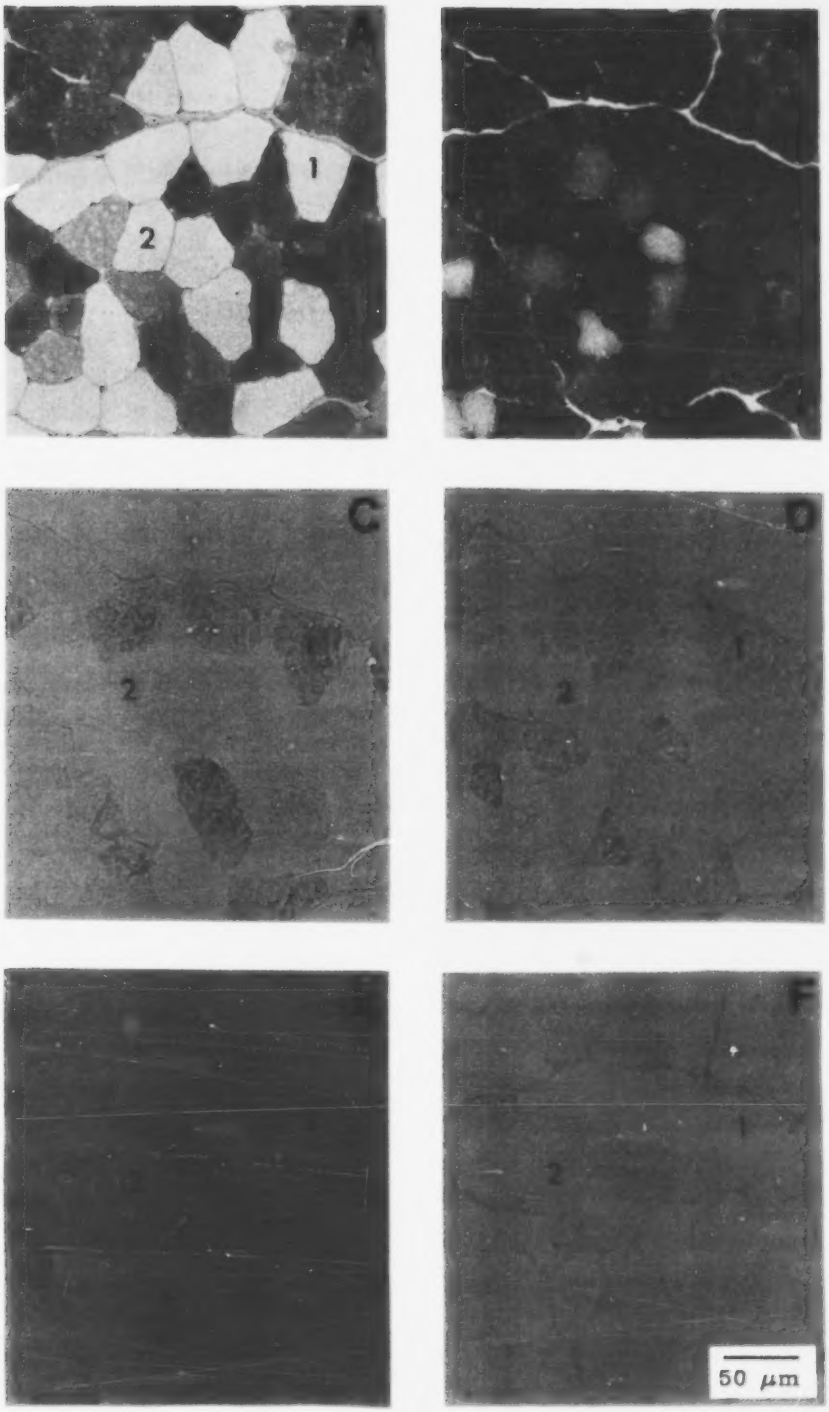
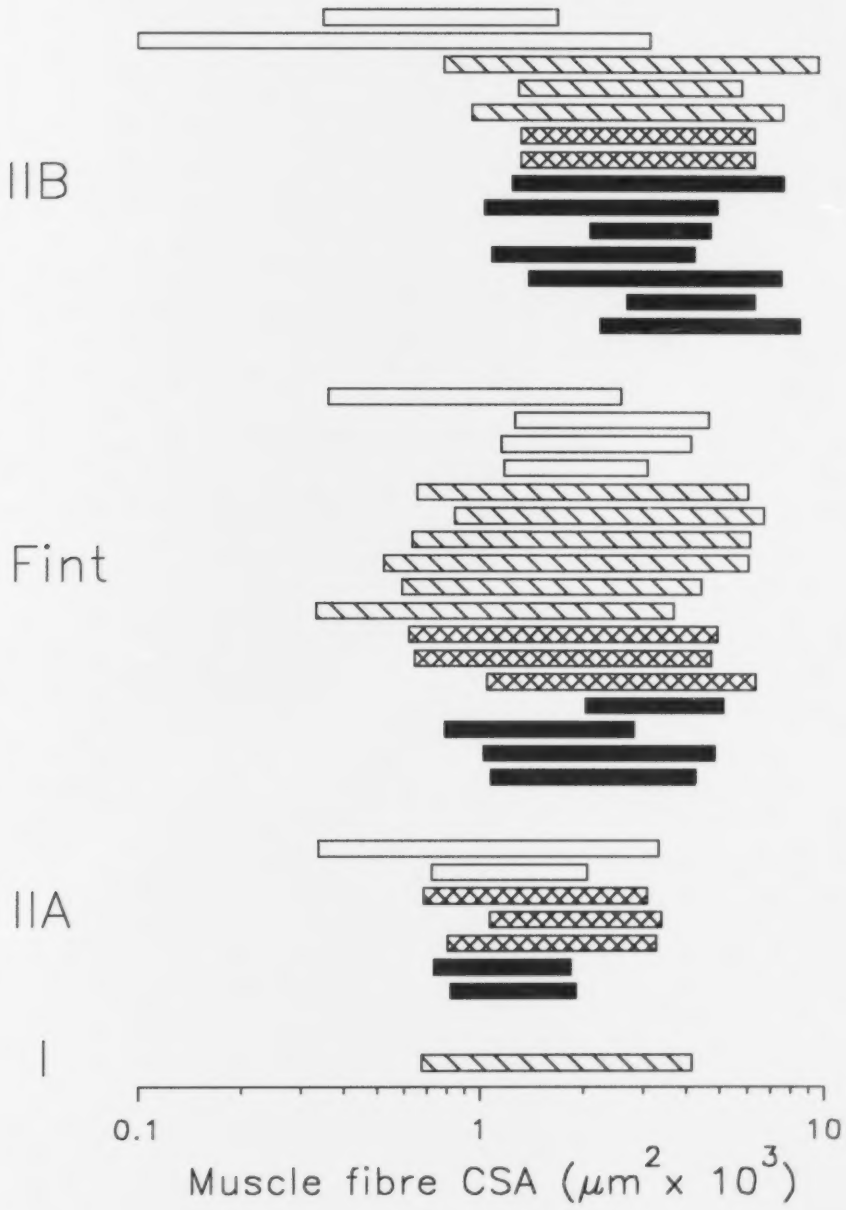

1
2
C
2
D
1
2
F
1
2
50 μm

Fig. 5.5

The range of MU muscle fibre cross-sectional area in glycogen-depleted MU in control (solid bars) and reinnervated muscles after immediate nerve repair (cross-hatched bars), after prolonged axotomy (hatched bars), and after prolonged denervation (open bars) prior to nerve repair. Note that the range of muscle fibre cross sectional area in reinnervated MUs was significantly larger than that of the control.


IIB
Fint
IIA
I
0.1
1
10
Muscle fibre CSA ($\mu m^2 \times 10^3$)

Chapter 6

General Discussion

6.I...MOTOR UNIT NUMBER

6.II...MOTOR UNIT SIZE

6.III...MUSCLE FIBRE SIZE

6.IV...MUSCLE PROPERTIES

6.V...BIBLIOGRAPHY

It is commonly observed that functional recovery after nerve repair deteriorates as a result of long distance between injury site and the target and/or long time elapse between nerve injury and repair. Several fundamental processes that may compromise axonal regeneration and muscle reinnervation are addressed in the present thesis. (1) The axotomized neurons progressively fail to regenerate and branch successfully. (2) Denervated nerve sheaths fail to provide trophic and substrate support for the regenerating nerve. (3) Denervated muscle fibres fail to survive, or accept reinnervation, or recover normal size and properties. Using a cross nerve anastomosis regime in rats, motoneurone axotomy and muscle/intramuscular nerve sheath denervations were independently varied. The present experiments examined the effects of immediate nerve cross suture, immediate nerve implantation onto denervated muscle surface and delayed nerve repair on the following parameters all of which are crucial for functional motor recovery: 1) the number of motor axons which successfully regenerate and make functional connections, 2) the capacity of regenerating axons to branch, 3) the recovery of muscle fibres from denervation atrophy, and 4) recovery of muscle properties after reinnervation. In addition, using the same cross-reinnervation paradigms, the extent of neural control over muscle fibre properties was evaluated.

6.I...MOTOR UNIT NUMBER

The number of functional MUs, critical for functional recovery of motor function, was complete after primary nerve-nerve cross suture (N-N suture). In contrast, MU

number decreased significantly when denervated muscle was neurotized by direct implantation of sectioned nerve on denervated muscle surface (N-M suture). The poorer recovery after N-M suture than after N-N suture suggests that denervated muscle surface is inferior to the intramuscular nerve sheaths in supporting nerve regeneration. This finding complements to studies showing that Schwann cells in the intramuscular nerve sheaths provide optimal support for regeneration (Glasby *et al.* 1986; Hall 1986a,b; review: Fawcett and Kenyes 1990). In the absence of Schwann cells, nerve and muscle basal laminae can only support axonal elongation for a short distance. Schwann cells migrating within the basal lamina of the nerve sheath, from either of the two nerve stumps, play a critical role in promoting axonal growth for long distance (Hall 1986a,b; review: Fawcett and Kenyes 1990).

Delayed nerve repair, commonly seen in clinical settings, is detrimental for functional recovery as demonstrated by the present experiments in which motoneurone axotomy and muscle denervation were independently varied. Even in an optimal environment with the presence of both Schwann cells and their basal lamina in freshly denervated nerve stumps, prolonged axotomy (>6 months) reduced the number of axons which regenerated and reinnervated the target muscle to 30% of that after primary nerve repair (Chapter 3). These findings suggest that growth-associated events after prolonged axotomy (such as upregulation of growth-related axonal molecules) may not be optimally maintained (Cassar *et al.* 1991; Cassar & Tetzlaff 1993). In addition, the capacity of

nerve regeneration may deteriorate as a result of severe atrophy of proximal axons (Gordon *et al.* 1991; Davis *et al.* 1978).

Regeneration was even more severely compromised after prolonged denervation of target muscle and intramuscular nerve sheaths than after prolonged axotomy. MU number further decreased to 10% of normal when denervation was prolonged beyond 180 days. This indicates that growth support provided by the distal endoneurial sheaths and denervated muscle fibres progressively deteriorates with prolonged denervation. Fragmentation of Schwann cell basal lamina and reduction in the number of viable Schwann cells in the intramuscular nerve sheaths are both likely to account for the waning growth support (Brunetti *et al.* 1985; Salonens *et al.* 1985 & 1987; Giannini & Dyck 1990).

In conclusion, the combined effects of prolonged motoneurone axotomy and prolonged distal nerve sheath/target muscle denervations, seen together after delayed nerve repair but separately evaluated in the present thesis, result in dismal functional recovery in terms of a small number of successfully regenerated axons.

6.II...MOTOR UNIT SIZE

The branching capacity of regenerating motor axons was rarely compromised under any experimental conditions imposed in the present thesis: prolonged motoneurone axotomy, prolonged muscle and distal nerve sheath denervations, and immediate nerve implantation onto muscle surface. Measurements of MU force and innervation ratio in

cross-reinnervated muscles show that regenerating axons branched extensively and reinnervated as many muscle fibres as intact nerve axons do in partial denervation to compensate for the reduction in the number of functional MUs. The increase in the number of muscle fibres per motoneurone (IR) has the same upper limit of 3-5 fold as intact neurons that sprout during partial denervation in cat tibialis anterior muscle (Brown & Ironton 1978; Rafuse *et al.* 1992). In the smaller rat muscles, branching capacity is not critically dependent on provision of nerve sheaths since MU enlargement was the same after direct neurotization (N-M suture) as after primary N-N suture. In addition, branching capacity was not reduced in delayed nerve repair. However, the upper limit of MU enlargement disallows complete compensation for too few MUs after prolonged muscle denervation.

6.III...MUSCLE FIBRE SIZE

Recovery of motor function also depends on the extent to which denervation atrophy of muscle fibres is reversed. After immediate nerve repair, complete formation of nerve-muscle contacts completely reverses denervation. The finding described in Chapter 3 (that reinnervated muscle fibres recover their normal size after long-term axotomy) shows that prolonged axotomy does not compromise this ability of motoneurones to reverse denervation atrophy. However, when long-term denervated, muscle fibres failed to recover completely in size even after reinnervation by a freshly cut nerve

(Chapter 4). Depletion of satellite cell population may account for the incomplete recovery in the size of reinnervated muscle fibres (Anzil & Wernig 1989).

6.IV...MUSCLE PROPERTIES

Recovery of motor function further depends on re-establishment of the normal wide range of MU forces and recovery of normal muscle fibre properties. After complete severance and repair, regenerating axons do not reinnervate their original muscle fibres and MU fibres tend to clump with more frequent adjacency consistent with fibre type grouping (Kugelberg *et al.* 1970; Totosy de Zepetnek *et al.* 1992a). This is in contrast with the mosaic distribution of normal MU muscle fibres which intermingle with fibres of other MUs. Initially, MU fibres are of several different types. With increasing time after reinnervation, the normal relationship between axon size, force, and speed returns as muscle fibres become homogeneous with respect to their histochemical profile (Gordon & Stein 1982a, b). This is true for both self- and cross-reinnervated muscles (Gordon *et al.* 1988). Thereby, the normal wide range of MU forces are eventually restored. Re-establishment of the normal range of MU forces primary depends on the difference in the number of muscle fibres reinnervated by different motoneurones since MU force is positively correlated with innervation ratio (Bodine *et al.* 1986; Totosy de Zepetnek *et al.* 1992a). Normally, MU force is determined by the mean muscle fibre cross-sectional area and innervation ratio (and to a much smaller extent by the specific force). Forces produced by reinnervated MUs depend much less on muscle fibre cross-sectional area and

specific force. This is explained by the finding that muscle fibres of different histochemical types become similar in size after reinnervation and variance among MU fibre CSA of different types of MUs is smaller than normal (Totosy de Zepetnek *et al.* 1992a). Therefore, the number of muscle fibres in each MU is the determinant of MU force and responsible for the observed wide range of MU forces in the reinnervated muscle. The number of muscle fibres each motoneurone innervates or the extent of branching is likely related to the size and activity of motoneurones since size-dependent branching is observed in both reinnervated and partially denervated muscles (Gordon *et al.* 1992; Rafuse *et al.* 1992).

After reinnervated by a single motoneurone for sufficient time, muscle fibres of different types become homogeneous in their histochemical profile (Kugelberg 1970; Totosy de Zepetnek *et al.* 1992a; Chapter 5). However, conventional histochemical staining of muscle fibres does not discriminate the higher variance in either myosin heavy chain isoforms or metabolic enzymes (Chapter 5; Sosedia *et al.* 1994). Using antibodies against different myosin heavy chain isoforms, we found that single reinnervated fast MUs contained muscle fibres which expressed different fast myosin heavy chain isoforms even long after reinnervation, consistent with previous finding in the cat (Gauthier *et al.* 1983; Unguez *et al.* 1993). Thus neural regulation over muscle fibre properties, though strong and possibly predominating as shown in the finding that fast MUs contain fibres expressing more than two types of MHC isoforms and many MUs are homogeneous (Pette & Vrbova 1992), is not complete (Chapter 5). In addition, long-term axotomy does not reduce the

observed neural determination of muscle fibre histochemical profile (Chapter 5). Regenerating axons also have a strong influence on the highly atrophic and regenerating muscle fibres that occurs after reinnervation in long-term denervated muscles (Chapter 4).

In summary, the regenerative capacity of motoneurones and the growth support provided in the distal intramuscular nerve sheaths and surface of denervated muscle decline rapidly after nerve injury. However, neither long-term axotomy nor long-term muscle and nerve sheath denervation compromises the capacity of regenerating motoneurones to reinnervate an increasing number of muscle fibres or to re-specify muscle fibre properties. Thus, the enhanced axonal branching can at least partly compensate for the overall reduction in the number of functional MUs. Prolonged denervation is detrimental to functional recovery because the number of functional MUs is largely reduced. This is further exacerbated by incomplete recovery of muscle fibres from denervation atrophy. Therefore, poor muscle recovery after delayed nerve repair is the result of combined effects of long-term muscle and distal nerve sheath denervation and long-term motoneurone axotomy.

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